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CORRECTION

The following captions should be substituted for those appearing below the figures in the paper *The adsorption of vitamin A by sodium bentonite*, by D. H. Laughland and W. E. J. Phillips, Can. J. Biochem. Physiol. 32 : 610-620. 1954.

FIG. 1. Adsorption of vitamin A alcohol, acetate, and palmitate on sodium bentonite.

– – – Alcohol; ——— Acetate; ····· Palmitate.

FIG. 2. Absorption curve of vitamin A alcohol and anhydro form.

– – – Vitamin A alcohol; ——— Anhydro form; ····· Mixture of equal weights of the two forms.

FIG. 3. Skellysolve B extracts of sodium bentonite treated with vitamin A.

– – – 1 min.; ——— 5 min.; ····· 15 min.; –·–·– 30 min.; –···– 120 min.

FIG. 4. Observed and calculated curves for a mixture of the alcohol and anhydro forms of vitamin A.

– – – Observed curve; ——— Calculated curve.

FIG. 5. Product extracted from bentonite.

——— Crude product; ····· Oxygenated product; – – – Recrystallized product.

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THE ACTION OF BENZIMIDAZOLE ON ENZYMATIC ADAPTATION[1]

BY DONN J. KUSHNER[2] AND ENID PASCOE

Abstract

The adaptive formation of formic hydrogenlyase in *E. coli* and of the enzymes oxidizing benzoate, malonate, and L-tyrosine in *P. fluorescens* is inhibited by benzimidazole. In one strain of *E. coli* tryptophan and alanine in combination completely reverse the benzimidazole inhibition. In another strain tryptophan partly reverses the inhibition but alanine is without effect. These amino acids are without effect on the benzimidazole inhibition of adaptation in *P. fluorescens*. Adenine and guanine have no action on the benzimidazole inhibition in both bacterial species.

Tryptophan disappears anaerobically in the presence of *E. coli*. Ammonia production accompanies tryptophan disappearance. This disappearance, which probably involves a destruction of the tryptophan molecule, is inhibited by benzimidazole.

Introduction

The observations of Woolley (7) that the inhibitory action of benzimidazole on the growth of several microorganisms could be reversed by the addition of adenine and guanine indicated that benzimidazole may act on bacterial growth as a purine antagonist. Pollock (3) found that benzimidazole was able to inhibit the adaptive formation of nitratase in nonproliferating cells of *Escherichia coli*, and Slonimsky (5) has recently reported a benzimidazole inhibition of the adaptive synthesis of respiratory enzymes in yeast. The present paper deals with the effect of benzimidazole on the adaptive formation of formic hydrogenlyase in *E. coli* and on the adaptive formation of enzymes oxidizing various substrates in *Pseudomonas fluorescens*. Benzimidazole has been found to act as an inhibitor of all adaptive processes studied. In inhibiting formic hydrogenlyase formation in *E. coli*, benzimidazole behaves not as a purine antagonist but as an amino acid antagonist.

Materials and Methods

The two strains of *E. coli* used, and the method of their growth and preparation for manometric experiments were the same as reported earlier (2). The strain of *Pseudomonas fluorescens* used was obtained through the courtesy

[1] *Manuscript received August 30, 1954.*
Contribution from the Research Institute, Montreal General Hospital, Montreal, Quebec. Supported by a grant from the National Research Council of Canada and by fellowships from The National Foundation for Infantile Paralysis and from Canada Packers Ltd.
[2] *Present address: Forest Insect Laboratory, Sault Ste. Marie, Ontario.*

of Dr. R. H. Wallace of the Department of Bacteriology of MacDonald College, McGill University. The organisms were grown overnight at 37°C. on medium of the following composition: 1% glucose, 0.5% NH_4Cl, 0.02% $MgSO_4.7H_2O$, 0.02% NaCl, 0.02% KCl, 1.5% Difco bacto-agar, and traces of $FeCl_3$ and $MnSO_4$ in equal volumes of 1% sodium phosphate buffer, pH 7.0, and tap water. The bacteria were washed three times in distilled water before use.

Standard manometric techniques were used for measuring formic hydrogenlyase activity (2) and bacterial respiration. The conditions for stimulating formic hydrogenlyase synthesis were essentially the same as those previously used (2), with minor variations as noted.

Tryptophan disappearance was measured by changes in the ultraviolet absorption. In some experiments benzimidazole was also present. A comparison of the ultraviolet absorption spectra of the two compounds showed that the absorption maximum of benzimidazole occurs at 270 mμ and that of tryptophan at 280 mμ. At 290 mμ, where the absorption by tryptophan is about half-maximal, benzimidazole has no measurable absorption. To eliminate benzimidazole interference, therefore, tryptophan absorption was measured at 290 mμ. By the use of appropriate controls, corrections were made for the ultraviolet-absorbing substances released by bacteria during incubation in the buffered medium used.

Indole was estimated qualitatively by Ehrlich's reaction. Ammonia was determined by the micro-diffusion method of Braganca, Quastel, and Schucher (1). All experiments were carried out at 37° C.

Results

The Effect of Benzimidazole on Formic Hydrogenlyase Formation in E. coli

In Strain 1 of *E. coli* when formic hydrogenlyase formation took place in the presence of a mixture of five amino acids (see Table I), with glucose as a source of both formate and of energy, benzimidazole inhibited adaptation. When the simplified amino acid mixture was replaced by peptone, by an acid hydrolyzate of casein supplemented with tryptophan, or by a synthetic mixture of the amino acids present in this hydrolyzate, the degree of enzyme formation was only slightly increased, but benzimidazole no longer had any significant inhibitory effect. These results showed that the inhibition by benzimidazole was reversible and that an amino acid or amino acids were involved in this reversal. By a systematic examination of the 20 amino acids in the complete mixture, it was found that only two, tryptophan and alanine, had any significant reversing effect on the benzimidazole inhibition. The reversal of the benzimidazole inhibition by these two amino acids as well as by a complete amino acid mixture is shown by the results given in Table I, experiments 1 and 2, and the complete reversal caused by tryptophan and alanine in combination is illustrated in Fig. 1. The benzimidazole inhibition appeared as an increase in lag periods, as a decrease in final rate of

hydrogen production, or both. As the figures presented in Table I show, there was a variation in the degree of inhibition found in different experiments, so that the reversals observed do not appear equally striking in all cases.

TABLE I

THE REVERSAL OF THE BENZIMIDAZOLE INHIBITION OF FORMIC HYDROGENLYASE SYNTHESIS IN *E. coli* BY AMINO ACIDS

Strain of *E. coli*	Expt.	Basic medium	Additions	Lag (min.)[4] T_1	Lag (min.)[4] T_2	Final Q_{H_2}
1	1	Glucose + five amino acids	Nil	50	90	651
			Benzimidazole	80	130	584
		Glucose + twenty amino acids	Nil	40	90	810
			Benzimidazole	50	90	876
	2	Glucose + five amino acids	Nil	60	100	331
			Benzimidazole	90	120	278
			Benzimidazole + alanine[1]	60	110	319
			Benzimidazole + tryptophan[1]	60	110	462
2	3	Glucose + formate[2] + seven amino acids	Nil	40	110	407
			Benzimidazole	50	120	216
		Glucose + formate + twenty amino acids	Nil	40	110	462
			Benzimidazole	40	120	296
	4	Glucose + formate + eight[3] amino acids	Nil	40	100	300
			Benzimidazole	70	80	55
			Alanine	40	100	302
			Alanine + benzimidazole	70	80	45
	5	Glucose + formate + eight amino acids	Nil	60	100	374
			Benzimidazole	80	120	154
			Tryptophan	60	120	494
			Tryptophan + benzimidazole	60	120	390

[1] *In this strain the addition of tryptophan and alanine had no effect on the lag and little effect on the final rate of hydrogen evolution. See Fig. 1.*

[2] *Formate was necessary for maximal adaptation in this strain. The substitution of glucose and formate for glucose in Strain 1 caused no change in the amino acid reversal of benzimidazole inhibition.*

[3] *In certain experiments it was found necessary to add threonine to the seven amino acid mixture to obtain maximal formic hydrogenlyase synthesis.*

[4] *Lag T_1 = Time of first appearance of activity.*
Lag T_2 = Time final steady rate of hydrogen evolution is reached.

CONDITIONS:

0.033M phosphate buffer, pH 7.4; 0.033M glucose; 0.067M sodium formate; 0.0033M benzimidazole with Strain 1; 0.003M benzimidazole with Strain 2.

Five amino acids: DL-serine, 1.67 mgm./ml.; L-histidine, 0.33 mgm./ml.; DL-aspartic acid, 1.4 mgm./ml.; L-leucine, 2.4 mgm./ml.; L-arginine, 0.65 mgm./ml.

Seven amino acids: Five amino acids + L-lysine, 1.0 mgm./ml.; DL-valine, 1.77 mgm./ml.

Eight amino acids: Seven amino acids + 1.5 mgm./ml. DL-threonine.

Twenty amino acids: The same amount per ml. as found in 0.33 ml. 5% casein hydrolyzate (according to data of Williamson (6)), with addition of 0.25 mgm./ml. DL-norleucine.

DL-alanine, 0.77 mgm./ml.; L-tryptophan, 0.22 mgm./ml.

1.5 mgm. bacteria in side arm, in 0.5 ml. H_2O tipped in after gassing with N_2 and equilibration. KOH in center well. Total volume, 3.0 ml.

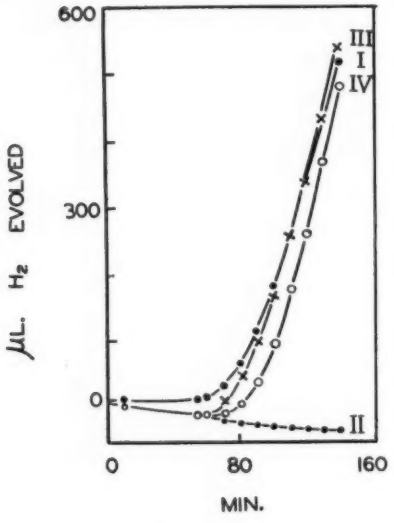

FIG 1. The reversal of the benzimidazole inhibition of formic hydrogenlyase synthesis by tryptophan and alanine.
Additions to glucose + five amino acid medium:
I, Nil;
II, Benzimidazole;
III, Tryptophan + alanine;
IV, Benzimidazole + tryptophan + alanine.
E. coli Strain 1, conditions as in Table I.

The properties of *E. coli* Strain 2 in regard to the benzimidazole inhibition and its reversal by different amino acids are also shown by the results given in Table I, experiments 3–5. In this strain, the presence of a mixture of 20 amino acids did not completely reverse the action of benzimidazole. Tryptophan was able to cause a partial reversal of inhibition, and alanine was without effect. The other amino acids, tested in groups of three or four, proved ineffective in reversing the benzimidazole inhibition.

Although a tryptophan concentration of 220 μgm./ml. was used in the experiments reported above, maximal reversal of the inhibition caused by 0.003*M* benzimidazole could be produced by 17 μgm./ml. and a significant reversal by as little as 1 μgm./ml. tryptophan.

Benzimidazole, in the concentrations used in these experiments, had no effect on the formic hydrogenlyase activity of adapted bacteria. The results indicate, therefore, an inhibitory effect on enzyme formation rather than on enzyme activity.

Since adenine and guanine are able partially to reverse the benzimidazole inhibition of growth, it was considered of interest to investigate the effects of these compounds on the benzimidazole inhibition of adaptation. It may

be seen from the results presented in Table II that neither adenine nor guanine was able to reverse the benzimidazole inhibition. From these findings, it appears unlikely that in inhibiting formic hydrogenlyase synthesis benzimidazole acts as a purine antagonist.

TABLE II

THE EFFECT OF ADENINE AND GUANINE ON THE BENZIMIDAZOLE INHIBITION OF FORMIC HYDROGENLYASE FORMATION

Additions to basic medium	Lag (min.) T_1	Lag (min.) T_2	Final Q_{H_2}
Nil	60	120	93
Benzimidazole	60	80	5
Adenine	60	120	92
Adenine + benzimidazole	60	80	1
Guanine	60	120	111
Guanine + benzimidazole	60	80	4

CONDITIONS:
Basic medium: Glucose + formate + seven amino acids, as in Table I. 0.33 mgm./ml. adenine sulphate; 0.1 mgm./ml. saturated guanine solution; 0.003M benzimidazole. Bacteria as in Table I (Strain 1).

No simple explanation of the reversing action of tryptophan and alanine is as yet possible. Special consideration was given to the role of benzimidazole as a tryptophan antagonist because of the structural resemblance of the two molecules and because tryptophan was found effective in reversing the benzimidazole inhibition in both strains of *E. coli* used. Although it was not found necessary to add tryptophan to obtain maximal formic hydrogenlyase formation, it is believed that tryptophan is used in the process of enzyme(s) formation, and that it is synthesized in sufficient amounts from the other amino acids present. Benzimidazole might act by inhibiting either the synthesis or the intracellular incorporation of tryptophan.

The uptake of tryptophan into bacterial cells from the external medium may serve in some measure as an index of intracellular tryptophan incorporation into proteins. Raff and Cohen (4) reported an incorporation of tryptophan into cells of *E. coli* incubated aerobically under conditions permitting growth. We have carried out experiments to determine whether such an incorporation took place during enzymatic adaptation, and, if so, if this incorporation were inhibited by benzimidazole.

The results of some of these experiments are presented in Table III. Anaerobic tryptophan disappearance takes place to approximately the same extent in cells suspended in a buffered medium without any added energy source as in those suspended in a medium containing glucose (as an energy source), formate, and the amino acids necessary to promote formic hydrogenlyase synthesis. No tryptophan disappearance was noted in the presence of boiled, washed cells or in cells incubated at 0° C. Since tryptophan

TABLE III

FACTORS AFFECTING THE DISAPPEARANCE OF TRYPTOPHAN IN THE PRESENCE OF *E. coli*

Expt.	μgm. tryptophan added	Other additions	μgm. tryptophan disappearing at 10 min.	20 min.	30 min.	60 min.	120 min.	180 min.
1*	100	Nil	8	12	12			
		Glucose + formate + eight amino acids	3	8	8			
2	100	Nil			25	25	42	
		Glucose + formate + eight amino acids			25	25	30	
3	50	Nil						30
		Benzimidazole						20
	100	Nil						35
		Benzimidazole						25
	300	Nil						52
		Benzimidazole						44

* *Mean results of three separate experiments.*

CONDITIONS:
E. coli Strain 1. Experiments were carried out anaerobically in tubes gassed with N_2. All tubes contained 3 mgm. bacteria, 0.033M phosphate buffer, pH 7.4, in a final volume of 3.0 ml. Other conditions were as indicated and as in Table I. At the times shown, tubes were cooled in ice, the bacteria centrifuged down at 0° C., and the absorption of the supernatant measured at 290 mμ.

disappearance apparently occurred without energy utilization it seems likely that an enzymatic destruction of the tryptophan molecule rather than an incorporation into the bacterial cell was taking place.

Further experiments on the presumed breakdown of tryptophan showed that indole formation from tryptophan takes place. Indole (measured by its ultraviolet absorption) also disappears anaerobically. The appearance of ammonia accompanies both tryptophan and indole disappearance (Table IV).

TABLE IV

THE PRODUCTION OF AMMONIA ACCOMPANYING TRYPTOPHAN AND INDOLE BREAKDOWN BY *E. coli*

Experiment	System	μgm. tryptophan or indole disappearing	μgm. ammonia produced
1	Tryptophan (1000 μgm.)	112	29.5
2	" (500 μgm.)	118	20.3
3	Indole (500 μgm.)	43	21.8
4	" (500 μgm.)	48	10.6

CONDITIONS:
E. coli strain 1. 0.033 M phosphate buffer, pH 7.4, 15 mgm. bacteria, tryptophan and indole as shown. Total volume 3.0 ml. Incubation under N_2 for 60 min.

Since the destruction of tryptophan can take place under conditions which do not permit adaptation to occur, it appears unlikely that it is intimately connected with formic hydrogenlyase synthesis. Whatever the relation, it should be noted that the disappearance of tryptophan is inhibited by benzimidazole (Table III). It is possible that because of their structural similarity benzimidazole and tryptophan mutually interfere with the accessibility of each other to the active sites on various enzymes. If this is the case, then in reversing the action of benzimidazole on formic hydrogenlyase synthesis, tryptophan may act simply by shielding from the toxic action of benzimidazole an enzyme that is concerned neither with tryptophan synthesis nor with its utilization.

The Action of Benzimidazole on Adaptive Enzyme Formation in P. fluorescens

Chief attention, in *P. fluorescens*, was given to the adaptive formation of enzymes catalyzing the oxidation of benzoate. From the data presented in Table V it may be seen that 0.003*M* benzimidazole caused a powerful inhibition of this adaptation. In contrast to the results obtained with *E. coli*, tryptophan produced no reversal of the benzimidazole inhibition. A complete amino acid mixture strongly inhibited the adaptation itself. Although both adenine and guanine had a stimulating effect on adaptation, they were unable to reverse the benzimidazole inhibition.

TABLE V

THE EFFECT OF AMINO ACIDS AND PURINES ON THE BENZIMIDAZOLE INHIBITION OF THE ADAPTIVE FORMATION OF ENZYMES OXIDIZING BENZOATE IN *P. fluorescens*

Expt.	Additions to 0.0013*M* benzoate	Lag (min.) T_1	Lag (min.) T_2	Final net Qo_2
1	Nil	—	—	21
	Benzimidazole	—	—	11
	Tryptophan	—	—	27
	Tryptophan + benzimidazole	—	—	9
2	Nil	—	—	23
	Twenty amino acids	—	—	1
3	Nil	220	240	22
	Benzimidazole	200	200	3
	Adenine	220	260	60
	Adenine + benzimidazole	200	200	6
	Guanine	220	260	72
	Guanine + benzimidazole	200	200	5

CONDITIONS:

Experiments 1 and 2 were carried out in 25 ml.Erlenmeyer flasks incubated aerobically with shaking for three hours. 0.033M phosphate buffer, pH 7.0, benzoate as indicated, tryptophan and amino acids in one third the concentrations used in Table I, 0.003M benzimidazole, 20 mgm. bacteria per flask, total volume 9.0 ml. At the end of the incubation the bacteria were washed, suspended in distilled water, and the benzoate oxidase activity of 1.5 mgm. lots assayed manometrically in the presence of 0.033M phosphate buffer, pH 7.0, and 0.0013M benzoate. Oxygen uptake was followed for 60–70 min., during which time no further adaptation took place.

Experiment 3 was carried out in Warburg vessels containing buffer, benzoate, and benzimidazole in the concentrations given above and adenine and guanine as in Table II. Three mgm. bacteria in 0.5 ml. H_2O were tipped in from the side arm after equilibration.

Only a few experiments could be carried out on the adaptive formation of enzymes oxidizing malonate and tyrosine, as the bacteria lost much of their ability to adapt to these substrates on continued cultivation. The experiments carried out showed, however, that both adaptive phenomena were inhibited by benzimidazole concentrations of 0.001*M* to 0.003*M* (Table VI). Benzimidazole had no inhibitory effect on the nonadaptive oxidations of malonate, benzoate, or tyrosine by bacteria grown in the presence of these substrates, nor did adenine or guanine affect the nonadaptive oxidation of benzoate.

TABLE VI

THE EFFECT OF BENZIMIDAZOLE ON THE ADAPTIVE FORMATION OF ENZYMES OXIDIZING MALONATE AND TRYOSINE IN *P. fluorescens*

Expt.	System	Lag (min.) T_1	Lag (min.) T_2	Final net Qo_2
1	Malonate	40	90	82
	Malonate + 0.001 *M* benzimidazole	90	—	9
2	Tyrosine	60	130	19
	Tyrosine + 0.001*M* benzimidazole	60	130	12
	Tyrosine + 0.003*M* benzimidazole	100	130	3

CONDITIONS:

0.033M phosphate buffer, pH 7.0, 0.0033M sodium malonate, 0.001M L-tyrosine, benzimidazole as indicated. Bacteria (in 0.5 ml. H_2O): Experiment 1, 1.5 mgm.; Experiment 2, 3 mgm. Bacteria tipped in from side arm after equilibration. Gas, air.

The experiments reported here, together with those of Pollock (3) and of Slonimsky (5), indicate that benzimidazole acts as a general inhibitor of adaptive enzyme formation in microorganisms. Slonimsky also found that adenine and guanine failed to reverse the inhibition of adaptation in yeast produced by benzimidazole. The available evidence, then, indicates that in inhibiting adaptation benzimidazole does not behave as a purine antagonist. In certain cases its action is reversed by amino acids, but the mechanism of this reversal is not yet clear.

Summary

1. Benzimidazole inhibits formic hydrogenlyase synthesis in nonproliferating suspensions of two strains of *E. coli*. In one strain the benzimidazole inhibition can be completely reversed by the presence of tryptophan and alanine. Other amino acids are without effect. In the second strain tryptophan causes a partial reversal of the benzimidazole inhibition but alanine is without effect.
2. Adenine and guanine, which antagonize the inhibitory action of benzimidazole on bacterial growth, have no effect on the benzimidazole inhibition of formic hydrogenlyase formation.

3. Tryptophan and indole are broken down anaerobically by *E. coli;* ammonia production accompanies the destruction of tryptophan and indole.
4. The anaerobic destruction of tryptophan is inhibited by benzimidazole.
5. Benzimidazole inhibits the adaptive formation of enzymes oxidizing malonate, benzoate, or tyrosine in nonproliferating suspensions of *Pseudomonas fluorescens*. The inhibitory effect of benzimidazole on the adaptation to oxidize benzoate is not reversed by tryptophan. A complete mixture of amino acids inhibits the adaptation itself.
6. Adenine and guanine stimulate adaptation but do not reverse the benzimidazole inhibition of adaptation in this organism.

Acknowledgment

The authors wish to express their gratitude to Professor J. H. Quastel for his interest and his helpful suggestions during the course of these studies.

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AN EFFECT OF ESTROGENS ON WATER INTAKE[1]

By J. S. Thompson

Abstract

Pellets of estradiol and cholesterol, when implanted subcutaneously, caused the expected urinary bladder dilatation and also an increase in water intake in male mice of the C57BL and A strains. Males of the C3H strain showed neither the bladder dilatation nor the increased water intake. The dilatation observed in the C57BL and A strains was at least partly dependent upon the increase in water intake, since limiting the water given to the estrogen-treated animals retarded the appearance and reduced the severity of the dilatation, and increased the survival rate.

Introduction

Dilatation of the urinary bladder in male mice of certain strains, when these animals are subjected to prolonged estrogen treatment, has been described by various authors including Shimkin *et al.* (4) and Horning (2). It was while investigating this phenomenon that the findings noted in this paper were observed.

Materials and Methods

The mice used in this investigation belonged to three well-known strains: the C57BL (Little), the A (Strong), and the C3H (Strong). Male mice of comparable age and weight of each strain were divided into control and experimental groups, kept in identical wooden cages, the same number of mice to a cage, and given Purina Fox Chow Checkers ad libitum. Water was also given ad libitum, except where otherwise noted, and the amount consumed was measured and recorded as cubic centimeters per mouse per day. The water bottles were interchanged between control and experimental cages from time to time, so that variations in the bottles or delivery tubes could not account for any apparent differences in consumption.

All the mice were examined by abdominal palpation at suitable intervals, to determine changes in the size of the urinary bladder.

Each mouse received a 5 mgm. pellet of either pure cholesterol (control group) or 20% estradiol[2] in cholesterol (experimental group). These pellets were prepared in the manner described by Shimkin (4) and were implanted subcutaneously through a small incision made in the skin of the back, with the aid of light ether anesthesia. After implantation the incision was closed with a wound clip.

Results

The estrogen-treated male animals of both A and C57BL strains showed a definite increase in water intake, which first started to appear 20 to 30 days after the pellet was implanted. The control animals did not show this

[1] *Manuscript received September 7, 1954.*
Contribution from the Department of Anatomy, University of Alberta, Edmonton, Alta. This work was carried out under Grant No. 111 of the National Cancer Institute.

[2] *The Ciba Company of Canada, Limited, supplied the estradiol and cholesterol used in this work.*

increase, although their treatment was identical to that given the experimental animals except for the difference in composition of the pellet. All the experimental animals of both strains showed simultaneously with the increased intake the dilatation of the urinary bladder mentioned in the introduction, this dilatation tending to appear and disappear in a cyclic manner. When fully dilated the bladder reached a point only one-half centimeter from the lower end of the sternum.

Neither estrogen-treated nor control male animals of the C3H strain showed an increase in fluid intake, nor did they show the dilatation of the urinary bladder found in the A and C57BL strains.

Table I compares the water intake of estrogen-treated and control mice of all three strains. In both series the figures indicate the average daily water intake for a period of at least 200 days. The measurements in the X series were commenced about 50 days after the pellets were implanted, by which time the increase is well established and therefore appears large. In the Y series, in which the measurements were commenced at the time of implantation, the increase appears to be somewhat smaller. However, both series show a significant increase (as determined by Fisher's t test) in the A and C57BL groups, whereas there is no increase in the C3H animals.

TABLE I

THE EFFECT OF ESTRADIOL UPON THE WATER INTAKE OF MALE MICE
(Intake expressed as cc./mouse/day)

Strain	Series	Estradiol	Control	Diff.	t	P
C57BL	X	7.1	4.2	2.9	27.79	<0.01
(50 animals)	Y	6.5	5.5	1.0	4.28	<0.01
A	X	7.9	4.8	3.1	7.16	<0.01
(76 animals)	Y	5.9	4.7	1.2	5.22	<0.01
C3H	X	6.7	6.9	0.2	0.75	0.4–0.5
(16 animals)						

To test the validity of these differences in water intake, two cages of apparently identical mice were set up on several different occasions and the water intake measured over varying periods of time (up to 150 days). At no time was there a significant difference in the intakes of two identical groups of mice. The results of two such tests are shown in Table II.

TABLE II

A COMPARISON OF THE WATER INTAKE OF GROUPS OF IDENTICAL MICE
(Intake expressed as cc./mouse/day)

Strain	Number of animals	Group X	Group Y	Diff.	t	P
A	15	4.94	4.62	0.32	1.96	0.1
C57BL	12	3.90	4.10	0.20	1.47	0.2

Those animals showing an increase in water intake also appeared to have a markedly increased urine output as judged by the wetness of the sawdust in their cages, that of the experimental groups being much wetter than that of the controls.

A and C57BL estrogen-treated animals lived for as long as 280 days after the urinary bladder dilatation appeared, but, in general, became less lively as time progressed, whereas the cholesterol-treated animals remained normal in this regard.

The number of animals to survive for 200 days after implantation in the A strain was 23 of 44 estrogen-treated and 29 of 32 controls. In the C57BL these figures were 18 of 25 estrogen-treated and 22 of 25 controls. The reduced survival rate of the estrogen-treated as compared to the controls is probably due to obstruction of the normal flow of urine in the former group.

Since the dilatation and increased water intake appeared in the same strains and at approximately the same time after implantation, it was decided to determine whether or not a relationship existed between these two phenomena.

Two groups of estrogen-treated male C57BL mice were set up, one of which, as a control, was given water ad libitum while the other received each day only 50–60% as much water as was consumed by the mice in the control cage.

Normally male C57BL mice show the dilatation of the bladder about 30 days after implantation of an estrogen pellet, but those mice whose water intake was forcibly reduced did not show dilatation for over 100 days, and then less markedly than the controls.

As well as reducing the degree of bladder dilatation, curtailing the intake also increased the relative number of estrogen-treated mice surviving for 200 days, as Table III shows.

TABLE III

THE EFFECT OF A FORCED REDUCTION IN WATER INTAKE UPON THE SURVIVAL OF ESTROGEN-TREATED C57BL MICE

Time of reduction	Survival time (days)	Survival rate					
		Low intake			Ad lib. intake		
		Orig. no.	No. surv.	% surv.	Orig. no.	No. surv.	% surv.
At implantation	250	10	9	90	18	10	55.5
After dilatation established	150	5	4	80	5	1	20.0

If the estrogen-treated animals were allowed to develop a very severe dilatation before the water intake was reduced, the dilatation became less marked after the reduction, but did not disappear. However, in these mice the relative number to survive for 150 days was also definitely increased, as Table III shows.

Both groups of animals on reduced intake appeared to be healthier and more lively than the controls.

Discussion

It is well known that estrogens tend to cause a retention of water in various tissues of the body (1), but the phenomenon herein reported obviously goes far beyond a matter of simple retention, involving as it does a very large increase in consumption as shown in Table I and some increase in excretion as shown by the wetness of the sawdust in the cages. These facts point to an increased excretion as well as an increased consumption of fluids in the estrogen-treated mice.

It should be stressed that this phenomenon occurs only in certain strains of mice and not in others. Light and Tornaben (3) have noted a similar increase in estrogen-treated rats.

The bladder dilatation has been reported by previous workers, but has never been satisfactorily explained (1), and may have a functional rather than an anatomical basis. The demonstration of a close relationship between the dilatation and the estrogen-produced increase in fluid intake tends to substantiate the conclusion that the dilatation is functional in origin.

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PHYSIOLOGICAL INVESTIGATIONS ON THE EXCRETION OF KYNURENINE IN HUMANS[1]

By M. Špaček

Abstract

Concentration of kynurenine in human urine shows variations of about 16% during the day and approximately as much (13%) from day to day. The variations are not clearly related to daily urine output, specific gravity, urinary creatinine, and the presence of indican or diazo-reaction.

Elimination of tryptophan from the diet leads to gradual decrease of kynurenine excretion in persons who have excreted large amounts on mixed diet, but not in persons excreting only traces. Concentration of kynurenine in urine rises after administration of tryptophan. The response to tryptophan varies widely from subject to subject. Nicotinamide seems to decrease excretion of kynurenine. No consistent effect of pyridoxine has been observed.

Older persons tend to excrete higher concentrations of kynurenine. Normal adults of 20 years average age present an average level of 0.1 mgm. kynurenine per 100 ml. of urine, much of which is not true kynurenine. The average level in older persons (47 years average age) without physical disease has been found to be 0.21 mgm. per 100 ml.

Introduction

Kynurenine has never been isolated from human urine but evidence has been advanced in the last few years that it may be present (1, 2, 5, 6).

No quantitative studies could be made until a convenient method became available (7). Such studies might be useful in the investigation of diseases.

The main object of this communication is to show which conditions may affect the significance of the analytical values. The influence of a few related compounds on the excretion of kynurenine was also studied, and the normal range of values for urinary kynurenine was determined.

Materials and Methods

Non-preserved specimens of urine were analyzed (whenever it is not stated otherwise, 24-hr. specimens were used). The normal range of values was determined on *(a)* physically well psychotics, *(b)* inmates of an old age institution, and *(c)* college students with no apparent disease. Other investigations were carried out on groups comprising both sick and healthy persons. Diet was standardized only in specific experiments, but its general character was noted as far as possible. Kynurenine was determined as described previously (7). Routine clinical procedures were used for determination of creatinine, pH, indican and diazo reaction.

Results

Variations of Concentration of Urinary Kynurenine During 24 hr.

The data collected in Table I were obtained on both sick and healthy persons on mixed diet. They should indicate the maximum variations to be

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expected in one and the same person. Since the fluctuations were large in only one case, it would appear unnecessary to carry out the studies on 24-hr. specimens. Collection of 24-hr. specimens was used in this work mainly in order to obtain enough material for other purposes.

TABLE I

VARIATIONS OF URINARY KYNURENINE (MGM. PER 100 ML.) IN DIFFERENT SPECIMENS OF URINE VOIDED

No. of exp.	During daytime						During night	Max. percentage deviation from average (%)
1138	0.37	0.47	0.48					15
1105	0.29	0.17	0.17	0.40				15
1122	0.43	0.37	0.40	0.47	0.49			14
1129	0.44	0.62	0.40	0.21	0.32	0.35		80
1126	1.10	1.05						5
1172	0.49						0.61	11
1177	0.10						0.14	15
1167	0.35						0.36	3
1167	0.39						0.39	0
1167	0.32						0.38	10
1167	0.61						0.38	15
1172	0.48						0.61	11
1177	0.12						0.10	7
1181	0.11						0.16	22

Average of the percentage deviations : 16%.

Variations of Urinary Kynurenine Over a Period of Four Days

As can be seen from Table II, values for one and the same person are not likely to change considerably if no obvious change has occurred in the patient's condition or diet.

TABLE II

VARIATIONS OF URINARY KYNURENINE (MGM. PER 100 ML.) OVER A FOUR DAY PERIOD

Exp. No.	1st day	2nd day	3rd day	4th day	Maximum percentage deviation from average (%)
1138	0.40	0.44			5
1105	0.35	0.25			17
1122	0.44	0.44			0
1129	0.43	0.37			8
1126	0.80	1.07			15
958	0.10	0.13	0.11	0.11	10
963	0.06	0.08	0.09	0.09	20
1167	0.35	0.39	0.35	0.54	23
877	0.74			1.25	25
654	1.45			1.30	5
921	0.39			0.47	10
915	0.44			0.34	11
798	0.16			0.20	11
726	0.18			0.13	14
723	0.45			0.31	18

Average of the percentage deviations : 13%.

Variations of Urinary Kynurenine With Specific Gravity and Volume

Daily urine output was measured in 74 persons and specific gravity in 32. It was expected that higher average kynurenine concentration would be found in groups with low urine output and high specific gravity, but statistical analysis failed to prove this convincingly.

Variations of Urinary Kynurenine With Urinary Creatinine

A survey of 65 cases showed that kynurenine excretion varies independently of the excretion of creatinine. This finding further supports the suggestion that concentration of kynurenine in urine does not primarily depend on diuresis.

Kynurenine and pH of Urine, Indican and Diazo Reaction.

Unusually high or low concentrations of kynurenine may be found in acid as well as alkaline urine. Diazo reaction was negative in a small series

TABLE III

URINARY KYNURENINE (MGM. PER 100 ML.) AFTER TRYPTOPHAN-FREE DIET. AVERAGE VALUES *(a)* WERE CALCULATED FOR CASES WHICH WERE FOLLOWED THE 1ST, 4TH, AND 6TH DAY, AVERAGE VALUES *(b)* FOR THOSE WHICH WERE EXAMINED ONLY THE 1ST AND 6TH DAY

Exp. No.	1st day	4th day	6th day	Note
436	1.51	0.75	0.36	
515	0.70	0.31	0.12	
641	0.44		0.15	Kynurenine above average on 1st day
394	0.35		0.26	
409	0.32	0.18	0.35	
431	0.27		0.27	
640	0.27		0.13	
482	0.25	0.15		
396	0.23		0.19	
442	0.23	0.19	0.17	
Average *(a)*	0.740	0.358	0.250	
Average *(b)*	0.479		0.222	
440	0.21	0.30	0.23	
437	0.21	0.17	0.16	
397	0.21		0.14	
435	0.20	0.18	0.16	
772	0.20	0.18		Kynurenine average or below on 1st day
408	0.18		0.18	
434	0.17	0.15	0.12	
439	0.15	0.32	0.21	
438	0.14	0.22	0.16	
443	0.13	0.05	0.14	
432	0.10	0.22	0.10	
410	0.08		0.11	
433	0.08	0.09	0.25	
411	0.06		0.11	
398	0.03		0.03	
Average *(a)*	0.176	0.189	0.175	
Average *(b)*	0.144		0.140	
Total average *(a)*	0.350	0.250	0.198	
Total average *(b)*	0.267		0.170	

including cases of extremely high and extremely low excretion of kynurenine. Indican reaction was quite often positive in urines containing large amounts of kynurenine, but also occasionally in those which contained only traces.

Urinary Kynurenine and Tryptophan in the Diet

Since kynurenine is a derivative of tryptophan, it should disappear rapidly from urine if no tryptophan is taken. In order to demonstrate this, 25 patients were kept for six days on a vegetarian regime, containing no milk, eggs, or cheese and only negligible amounts of meat (bacon). Before the experiment (Table III) urinary kynurenine showed a wide subject-to-subject variation although all the patients were on a similar mixed diet. As expected, the average concentration of kynurenine decreased after four days and, unexpectedly, still further after six days. This would suggest that not all of the urinary kynurenine is derived directly from the dietary tryptophan. The effect of tryptophan restriction was somewhat different in two groups of patients. Urinary kynurenine decreased significantly after six days (even after four days) in eight of the 10 persons who had excreted more than the average on mixed diet. In all but one of the others (significant increase) the change was so small that even the significance of the average slight decrease is doubtful. One is inclined to conclude that elimination of tryptophan from the diet makes little difference where there has been no tendency to excrete kynurenine, whereas in cases with abnormal values on mixed diet the excretion tends to return to normal.

Comparatively large doses of tryptophan were needed to affect urinary kynurenine; yet the doses used were not large enough to interfere with the determination of kynurenine (6, 7), although much of the dietary tryptophan is known to be excreted unchanged (4). Table IV shows the expected rise

TABLE IV

URINARY KYNURENINE (MGM. PER 100 ML.) AFTER ADMINISTRATION OF TRYPTOPHAN

Exp. No.	Before tryptophan	0–12 hr. after tryptophan	Dose of tryptophan, gm.	Note
877	0.74	10.0	3	D, L-Tryptophan
1160	0.22	2.60	2	D, L-Tryptophan
1160	0.24	2.40	2	D, L-Tryptophan; duplicate exp.
110	0.21	1.42	2	D, L-Tryptophan
111	0.18	1.12	2	D, L-Tryptophan
394	0.35	3.15	2.5	L-Tryptophan
408	0.03	1.52	1	L-Tryptophan
915	0.44	0.75	1.5	L-Tryptophan
2024	0.05	0.29	1.5	L-Tryptophan
512	0.10	0.24	1.5	L-Tryptophan
510	0.10	0.33	1.5	L-Tryptophan
916	0.13	0.37	1.5	L-Tryptophan
529	0.16	0.09	1.5	L-Tryptophan
743	0.29	0.37	1.5	L-Tryptophan
942	0.30	2.00	1.5	L-Tryptophan
580	0.14	0.56	1.5	L-Tryptophan

of urinary kynurenine after tryptophan but also a conspicuously large subject-to-subject variation in response to the same dose. Marked increase of kynurenine concentration did not always correspond with a high level on mixed diet and vice versa. The differences were not due to varying diuresis.

Influence of Vitamins

Nicotinamide and pyridoxine seemed most likely to affect the excretion of kynurenine. The vitamins were as a rule administered 36 hr. after the test dose of tryptophan; nicotinamide was usually given in three doses of 150 mgm. each and urine was collected for 24 hr. after the last dose. Pyridoxine was administered subcutaneously in a similar manner. Results shown in Tables V and VI demonstrate no consistent effect of pyridoxine. On the other hand, there was a fairly consistent decrease of urinary kynurenine after nicotinamide. This appears to be significant statistically, although it may be objected that the figures are too close to the blank value of urine so that the accuracy of the individual determinations may be insufficient.

TABLE V

INFLUENCE OF NICOTINAMIDE ON THE EXCRETION OF KYNURENINE (MGM. PER 100 ML.)

Exp. No.	Kynurenine before nicotinamide	Kynurenine after nicotinamide	Dose of nicotinamide, mgm.	Note
772	0.20	0.16	150	
482	0.25	0.11	150	
726	0.28	0.05	150	
525	0.07	0.12	300	child
486	0.26	0.20	450	child
489	0.19	0.16	450	
523	0.10	0.07	450	
527	0.13	0.09	450	child
532	0.11	0.08	450	child
512	0.10	0.07	450	child
743	0.29	0.29	450	
876	0.0	0.14	600	

TABLE VI

INFLUENCE OF PYRIDOXINE ON THE EXCRETION OF KYNURENINE (MGM. PER 100 ML.)

Exp. No.	Kynurenine before pyridoxine	Kynurenine after pyridoxine	Dose of pyridoxine, mgm.
985	0.08	0.12	200
877	0.74	0.66	300
436	1.71	1.88	300
998	0.10	0.07	500
515	0.70	0.88	500
574	1.90	0.79	800

TABLE VII

INFLUENCE OF AGE AND SEX ON URINARY KYNURENINE

(Kynurenine in mgm. per 100 ml., M—male, F—female; no distinction is made under 10 yr. of age)

	<10 yr. M and F	10–20 yr. M	10–20 yr. F	20–30 yr. M	20–30 yr. F	30–40 yr. M	30–40 yr. F	40–50 yr. M	40–50 yr. F	50–60 yr. M	50–60 yr. F	60–70 yr. M	60–70 yr. F	>70 yr. M	>70 yr. F
Average kynurenine	0.067	0.095	0.107	0.253	0.222	0.206	0.315	0.210	0.283	0.280	0.262	0.250	0.188	0.333	0.268
Number of cases	11	6	11	8	8	8	9	11	8	12	14	17	11	10	14
Average kynurenine for both sexes	0.067	0.102		0.236		0.260		0.240		0.272		0.225		0.295	
Total number of cases	11	17		16		17		19		26		28		24	

TABLE VIII

NORMAL VALUES FOR URINARY KYNURENINE IN ADULTS (MGM. PER 100 ML.)

	Average age 20 yr.	Average age 47 yr.
Kynurenine	0.096	0.207
Mean deviation from the average	±0.041	±0.112
Number of observations	68	24

Influence of Age and Sex

No marked difference between the two sexes can be observed in Table VII. This table includes many pathological cases, and rather more in the older age groups. However, the trend to increasing concentration of kynurenine with increasing age was confirmed on 92 physically healthy persons (Table VIII). In fact, many of the younger group (20 years) did not present sufficient evidence of any kynurenine and their average figure represents probably the blank value of urine more closely than true kynurenine. The age of the patient will affect the interpretation of results in disease (8).

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THE ACTIONS OF α-TOCOPHEROL ON MAMMALIAN HEARTS[1]

By F. C. Lu, M. G. Allmark, and W. D. Graham

Abstract

The effects of α-tocopherol were studied in a large number of experiments using isolated perfused mammalian hearts as well as the dog heart *in situ*. Under these conditions no significant pharmacodynamic actions of this agent, either on the coronary flow or the heart contraction, were demonstrable.

Since α-tocopherol was reported as useful in the treatment of various heart diseases (9, 10), its therapeutic value has been a matter of controversy. That α-tocopherol acts by virtue of a specific pharmacodynamic action on the heart, rather than as a replacement therapy, has been suggested to explain (1) its reported usefulness in the multiple types of heart diseases and (2) the necessity of administering fairly large doses of this substance (6). No experimental evidence was available to substantiate this suggestion until the publication of a paper by Vacarri (7) who reported that α-tocopherol (its phosphate ester) induces in the isolated rabbit heart an increase in coronary flow and an augmentation of heart beat when its concentration in the perfusate is 10^{-8} or lower. Higher concentrations, however, were reported to have deleterious effects. The observation that the optimum concentration of α-tocopherol was much lower in the isolated perfused rabbit heart than that required clinically was attributed by Vacarri to the absence in the perfusate of interfering substances normally present in the blood.

In the past few years a number of studies have been made by the author and co-workers on the coronary and cardiac effects of various drugs. During the course of these studies an investigation on the actions of α-tocopherol was made in an attempt to confirm or disprove the results reported by Vacarri. The experimental findings on this problem are presented in this paper.

Methods

The apparatus and procedure employed for the isolated perfused heart experiments have been described previously (4). With this apparatus the heart contraction and the total coronary inflow were recorded simultaneously. In the experiments on the dog heart *in situ* the blood flow through the cannulated anterior descending branch of the left coronary artery was recorded. The detail of the procedure may be found elsewhere (3). Approximately 100 tests in 30 isolated hearts of rabbits and other mammals and 10 tests in four dog hearts *in situ* were performed.

Five different α-tocopherol preparations were used: (1) *dl*-α-tocopheryl phosphate, (2) *dl*-α-tocopheryl acetate, (3) *d*-α-tocopheryl succinate, (4)

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Contribution from Food and Drug Laboratories, Department of National Health and Welfare, Ottawa, Ontario.

d-α-tocopheryl phosphate, and (5) "water-soluble α-tocopherol". The chemical composition of the last preparation was not disclosed by the manufacturer. The first two preparations were obtained from Hoffmann-La Roche, Inc., and the last three from Distillation Product Industries.

Results

In approximately half the experiments, the α-tocopherol was given as a continuous infusion. Six concentrations (ranging from 10^{-9} to 10^{-4}) were used in these experiments. For the two highest concentrations (10^{-5} and 10^{-4}) the water-soluble α-tocopherol only was used. The infusion period was usually between 5 and 10 min. In all these experiments there was no significant change in the rate of coronary flow or the amplitude of heart contraction. The tracings of two typical experiments are shown in Fig. 1(*A* and *B*). The ineffectiveness of α-tocopherol may be clearly seen from these tracings.

Single injections of α-tocopherol at doses ranging from 0.1 μgm. to 10 mgm. (at 10-fold intervals) were made in the other experiments. There was no appreciable change in either the rate or the amplitude of heart contraction. Moreover, increases in coronary flow were not observed in any of these experiments. However, a slight decrease in the rate of coronary flow was frequently seen, especially when large doses were given. This was evidently a result of the high viscosity of the solution injected. The tracing of a typical experiment is shown in Fig. 3(*A*).

While usually no myocardial or coronary effects were demonstrable after injections of α-tocopherol, a very slight increase in the amplitude of heart beat was observed in four experiments performed on two hearts. The tracings of two such experiments on one heart are shown in Fig. 3(*B* and *C*). It may be noted that even a thousandfold increase in the dose did not appreciably augment this slight cardiac effect. It is doubtful that this is of any significance in view of the slightness of the effect and the infrequency of its occurrence.

When an excessive dose (10 mgm.) of the less soluble esters of α-tocopherol was injected, a marked initial augmentation of heart contraction and a striking decrease in coronary flow were observed. The tracing of a typical experiment is shown in Fig. 2(*A*). The amplitude of heart beat as measured on the original tracing was 10 mm. before the injection and 19 mm. after the injection. The rate of coronary flow during the same period of time decreased from 10.1 cc. per min. to 1.8 cc. per min. The decrease in coronary flow was evidently a result of blockade of the coronary vessels by the undissolved particles of α-tocopherol ester; the augmentation of heart contraction was only a secondary effect owing to the partial coronary occlusion. Earlier studies have shown that a decrease in coronary flow resulting from either mechanical intervention (lowering of perfusion pressure or discontinuation of perfusion) (1) or injections of coronary constrictor agent (2) usually causes a marked initial augmentation and a delayed depression of heart contraction. The afore-mentioned changes may conceivably be due to some similar mechanism.

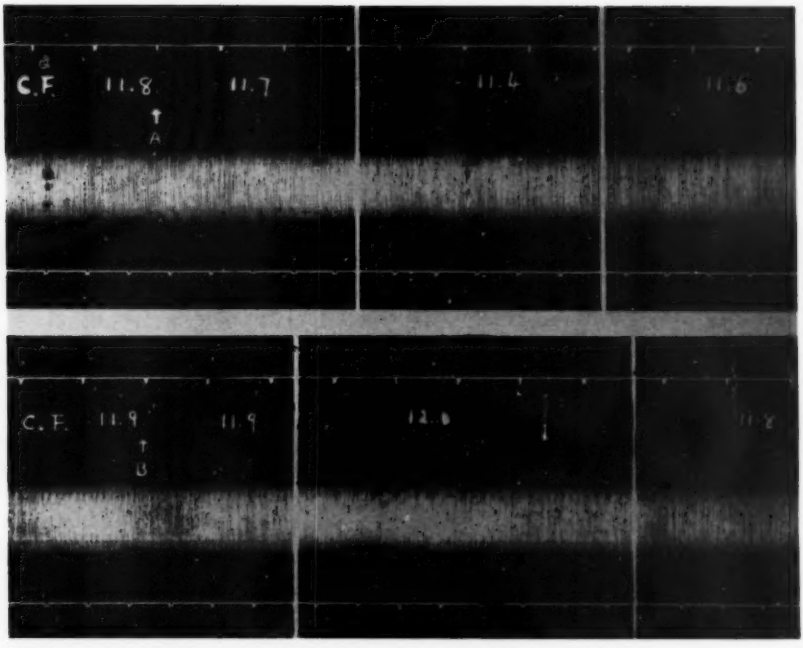

FIG. 1. Isolated perfused rabbit heart experiment.

In this and the following figures the tracings in each section are, from top to bottom; (1) coronary inflow, each interval represents the time at which 4 cc. of perfusate has entered the coronary vessels—the figures (C.F.) indicate the rate of coronary flow calculated in cc./min.; (2) heart contraction—systole, upstroke; (3) time, each interval represents 10 sec.—the kymograph was stopped for two minutes at each solid vertical line.

An infusion of α-tocopheryl phosphate was started at *A* and *B* at concentrations of 10^{-8} and 10^{-6} respectively.

FIG. 2. Isolated perfused rabbit heart experiment.

An injection of 10 mgm. α-tocopheryl phosphate was made at *A*.

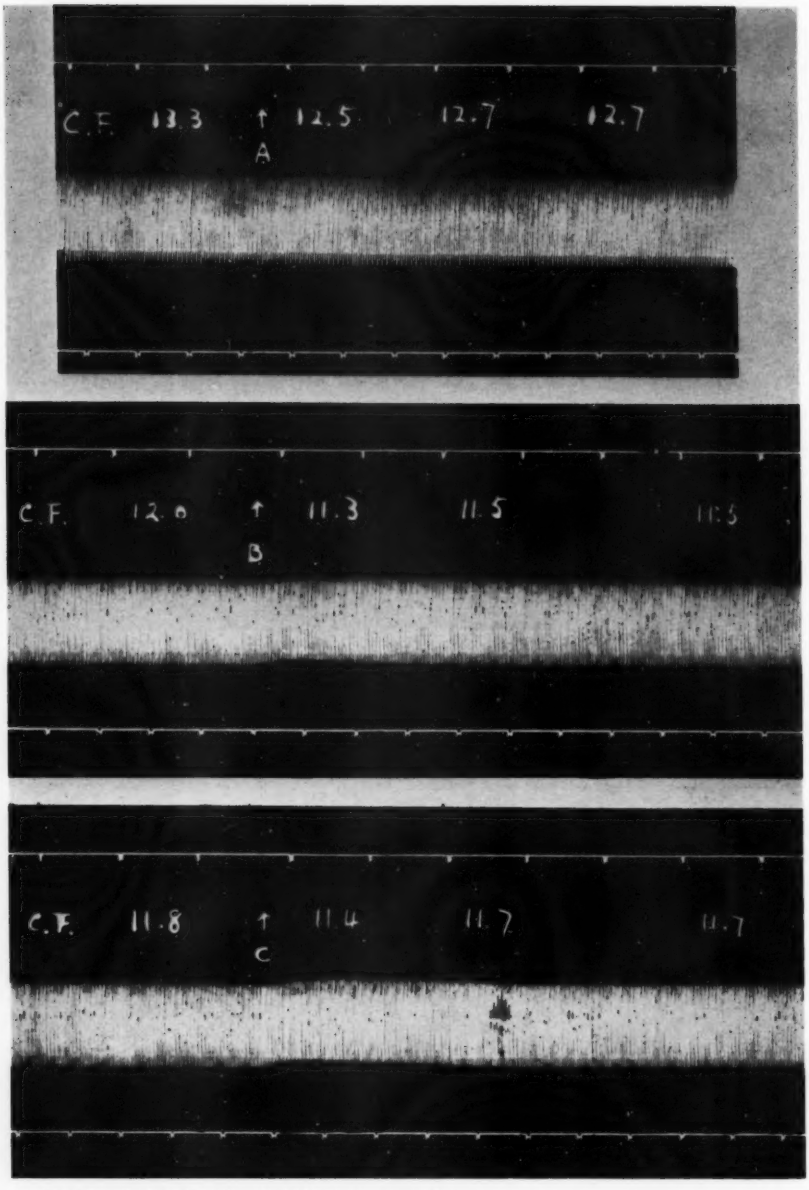

FIG. 3. Isolated perfused rabbit heart experiment.

Injections of 0.1 mgm., 1 μgm., and 1.0 mgm. of α-tocopheryl phosphate were made at *A*, *B*, and *C* respectively.

Since appreciable specific pharmacodynamic actions were not demonstrated with normal hearts of young adult rabbits, further experiments were performed on (1) hearts of two young (one month) and two old (two year) rabbits, (2) two hypodynamic hearts (one resulting from prolonged perfusion with oxygenated Locke's solution and the other from perfusion with nitrogen-saturated Locke's solution), (3) hearts of four rabbits fed with an α-tocopherol-deficient diet (5) for three to six weeks, (4) hearts of two dogs, three cats, two guinea pigs, and one rat, and (5) four dog hearts *in situ*. Furthermore, different pressures (ranging from 40 to 150 cm. H_2O) were used for the perfusion of the isolated hearts. It may be briefly stated that in none of these experiments was there any noticeable effect on the heart contraction or the coronary flow. Since no increase in coronary flow was observed in any of these experiments, the data were not analyzed statistically.

Discussion

From the foregoing experiments it is evident that α-tocopherol has no coronary dilator action or any significant stimulating action on the myocardium. These conclusions are based on results obtained under a wide variety of experimental conditions, including the use of several different preparations of α-tocopherol. These results do not agree with the observation of a coronary dilator activity in three of four rabbit heart experiments as reported by Vacarri (8). The reason for this disagreement is not clear.

The data would also suggest that the positive clinical results reported by some clinicians might be due to some action of the drug other than its direct effects on the coronary circulation or heart action. There is however no known evidence in support of any such extracardiac action of this agent.

It might be argued that the therapeutic effects are obtainable only after prolonged administration of α-tocopherol but are not demonstrable in acute experiments. However, this is probably unlikely in view of the fact that responses of isolated mammalian hearts are usually observed immediately after single injections of many drugs the full clinical effects of which are obtained only with prolonged administration. Digitoxin may be cited as an example of such drugs.

Finally, it is well known that animals maintained on an α-tocopherol-deficient diet develop certain degenerative changes in the heart. These pathological changes may be prevented by the administration of α-tocopherol. However, in view of the absence of specific pharmacodynamic actions even in animals suffering from α-tocopherol-deficiency, its curative value in heart diseases appears to be doubtful.

Acknowledgment

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THE LIPOTROPIC ACTIVITY OF PROTEIN[1]

By C. C. Lucas and Jessie H. Ridout

Abstract

A distinct lipotropic effect of casein has been observed in rats under conditions that appear to preclude any explanation involving the methionine or choline content of the ration. Diets low in protein produce a type of fatty liver that is not prevented by choline and is histologically different from that of choline deficiency. Choline appears less effective in such diets than in ones more adequate in protein. Presumably choline has only a limited effect on abnormal fat in periportal regions because the biochemical basis of this lesion seems to involve faulty formation of lipoprotein due to shortage of essential amino acids.

The occurrence of fatty livers in gluttons and drunkards as well as in patients suffering from infections or in persons poisoned by certain toxic chemicals has been recognized for several hundred years. Some advance in our understanding of the former, i.e., dietary, type of fatty liver was made in 1932 when it was discovered (8, 9) that an excessive accumulation of fat occurs in the livers of rats suffering from a specific dietary deficiency—lack of adequate choline. The amount of protein in the diet was soon found to affect the outcome of lipotropic studies (5, 6, 10, 20). The preliminary studies of Channon and his colleagues (2, 3, 4), who tested a number of amino acids in an attempt to account for the variable but sometimes pronounced effect of protein, failed to provide an explanation for the lipotropic activity of casein. They showed that under certain dietary conditions tyrosine exerted a questionable activity and that cystine produced an antilipotropic effect. The lipotropic property of proteins seemed to be accounted for in 1937 when Tucker and Eckstein (41) discovered that methionine possesses marked lipotropic activity. This subject soon received further study in several laboratories (13, 14, 15, 17, 18, 19, 30, 37, 40, 42, 43).

Channon *et al.* (17) found considerable variations in the lipotropic effect of different proteins. Tucker and Eckstein (42) pointed out that the relative activities of these proteins were in most cases approximately proportional to the methionine content. Best and Ridout (13) noted that the lipotropic activities of D- and L-methionine were of the same order. Their studies indicated that other factors, in addition to cystine and methionine, may be involved in determining the lipotropic effect of a protein. This idea, previously considered by Tucker and Eckstein, became increasingly probable as the following observations were accumulated. Channon, Manifold, and Platt (18) independently confirmed the observations of Best and Ridout that supplementary casein exerts a distinctly stronger lipotropic effect than does a supplement containing amounts of free methionine and cystine exactly equal to those contained in the casein. Eckstein and his colleagues (30,

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40, 43) found just the opposite. Beveridge, Lucas, and O'Grady (15) pointed out five differences in the experimental conditions used by the two groups of investigators and showed that the level of dietary protein at which the comparison is made is important. The adequacy of the protein in the basal diet will affect the rate of growth and thus the amount of choline diverted for structural purposes and also the amount of methionine so used (cf. Griffith *et al.* 23, 25, 26, 33). The data of Beveridge *et al.* (14, 15) led them to conclude that the lipotropic activity of a protein is determined not only by its content of methionine and cystine, but also by the nature and quantity of the essential amino acids contained in it. They presented further evidence, nebulous it is true, for the existence in casein of a lipotropic factor other than methionine, possibly tyrosine (cf. 3, 4). The role of tyrosine is still ambiguous.

Strangely enough, no experiment concerning the lipotropic effect of protein ever seems to have been conducted in which there were not several uncontrolled variables. In an attempt to reduce these undesirable features to a minimum the following simple experiment has been conducted and a distinct lipotropic effect of protein, not related to the methionine or vitamin B_{12} content of the diet, has been observed.

Methods

Groups of six male rats (Wistar strain, 60 to 80 gm.), in individual cages, were fed the test diets ad libitum for three weeks. Care of the animals and analytical procedures have been described fully elsewhere (11, 12).

Four diets containing, respectively, 3, 6, 9, and 18% of vitamin-free casein (General Biochemicals) were prepared in which the total methionine was kept uniform at 0.54% (equivalent to that in the diet containing 18% casein), by adding suitable amounts of DL-methionine. In percentage, the composition of the rations was salt mixture* 3, celluflour 2, sucrose–vitamin mixture* 1, corn oil 5, cod liver oil concentrate* 0.010, α-tocopheryl acetate 0.010, choline chloride 0.12, and finely powdered sucrose to make up to 100. All diets contained also 3 μgm. of vitamin B_{12} per 100 gm. Thus all diets contained exactly equal amounts of choline and known choline-precursors.

Results and Discussion

More fat was found in the livers of the rats on diets low in protein than in those on more adequate rations (Fig. 1). In spite of the presence of the same amount of choline, methionine, and vitamin B_{12} respectively, in all the rations, a marked reduction in liver lipids occurred as the protein content of the diet was increased. Thus one inevitable conclusion appears to be that protein exerts a lipotropic effect which is mediated by some process not directly involving choline or its known precursors. Another conclusion to be drawn from the same data is that a given amount of choline is less able to maintain the liver lipids at normal concentrations in animals fed diets low in protein

* *Described in reference (12).*

than in those fed more adequate rations. A distinct but less pronounced lipotropic effect of protein is seen in similar diets free from choline and also when adequate choline is present (Fig. 2).

Histological examination of sections of these livers, by Professor Hartroft, revealed that in the rats fed low protein diets (containing added methionine and a little choline chloride), the bulk of the stainable fat was present around the periphery of the liver lobules, i.e. in regions adjacent to the interlobular vein, which carries a mixture of portal and arterial blood.

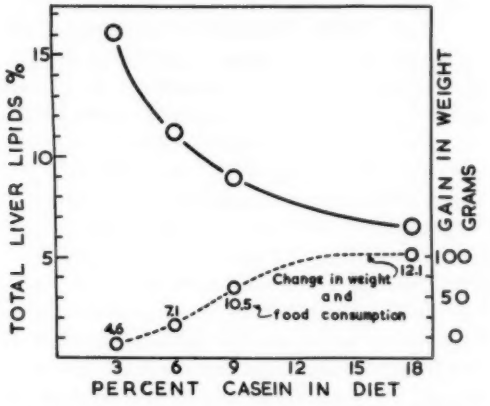

FIG. 1. Effect of the amount of protein in the diet on total liver lipids when the amounts of known lipotropic factors were kept constant: choline chloride 0.12%, total methionine 0.54%, vitamin B_{12} 3 μgm. per 100 gm. ration.

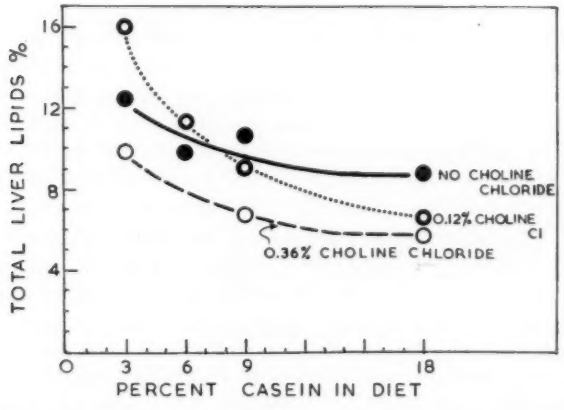

FIG. 2. Effect of different amounts of protein in the diet on total liver lipids when known lipotropic agents were kept constant at three different levels: total methionine always 0.54%, vitamin B_{12} always 3 μgm. per 100 gm. ration, choline chloride varied.

This periportal distribution of stainable lipid is different from that seen in choline deficiency; the fat appears first around the central vein when the generally accepted lipotropic agents (choline, betaine, and methionine) are inadequate. A more detailed comparison of the histological findings in fatty livers due to protein inadequacy with those due to choline deficiency is being published elsewhere by Best, Hartroft, Lucas, and Ridout (7).

A number of recent papers (e.g. 1, 21, 27, 28, 29, 31, 32, 34, 38, 39, and others) have indicated that dietary factors other than choline and its precursors affect the deposition of fat in the liver. It has been reported that some of these fatty livers respond only partially, and others scarcely at all, to added choline. Some appear to respond to threonine (21, 27, 28, 29, 38, 39) although under other conditions a small supplement of this amino acid can produce a fatty liver (24). It is well known that diets consisting largely of corn meal produce in children a malignant malnutrition (kwashiorkor) involving fatty livers that do not respond fully to the presence of choline. An excellent review of this subject is now available (16). Prolonged use of the same type of diet caused severe hepatic injury in rats (22). Shils and Stewart (35, 36) have pointed out that the stainable fat in the livers of rats fed diets consisting principally of corn meal is periportal in distribution, thus resembling the findings in children suffering from kwashiorkor. A suspicion that the periportal lesion might be due to some unique property of corn meal or corn proteins has had to be abandoned since we have now seen periportal fat (relatively unresponsive to choline) in rats fed low protein diets of many kinds, e.g. containing casein (as reported here), soya bean protein, fibrin, peanut protein, and various mixtures of these (7).

Until these observations were made it seemed worth while to try to isolate and identify the substance in casein responsible for the prevention of the fatty liver not responsive to choline. A review of the literature indicates that practically all of the situations in which fatty livers unresponsive to choline have been observed are characterized by diets low in protein. These facts and our own findings now make it doubtful that any isolatable factor is involved. It appears rather that adequate dietary protein is necessary to prevent some appearance of abnormal (stainable) fat in periportal regions. The amount of total lipids (extractable with hot alcohol) is not always raised significantly above the normal range when the amount of stainable fat in periportal regions is quite appreciable. Sometimes it is, however (7).

Dick *et al.* (21) have offered a suggestion with which we concur fully, viz: that some fatty livers may represent a nonspecific effect of protein deficiency rather than a specific effect of lack of any one essential amino acid (or unsuspected new constituent of proteins). Inadequate dietary protein (or in certain cases shortages of one or two essential amino acids) may lead to faulty production of protein in the cells of the liver or to faulty formation of the lipoprotein complexes characteristic of this tissue. Supplementary choline cannot, of course, make good a deficiency of essential amino acids. Hence it is ineffective in this type of fatty liver.

One might try to explain the results by assuming a greater effectiveness of methionine as choline-precursor in the diets richer in protein, on the basis of greater wastage of *free* methionine in the rations low in protein because of absorption of some of the methionine before other amino acids have been liberated from the protein. There is, however, no reason to believe that simultaneous availability of other amino acids is necessary for the biosynthesis of choline from methionine.

Protein contributes to protection of the liver in at least three ways: (1) by supplying methionine which, by providing methyl groups for formation of choline, prevents accumulation of excessive amounts of fat in the centrolobular regions, (2) by exerting a lipotropic effect which is independent of the methionine content and which seems to be exerted specifically in the periportal regions, (3) by providing the building blocks (essential amino acids) necessary for the maintenance and repair of the liver cells. It should be obvious that supplements of methionine alone can exert only limited benefit since methionine does not supply the factors concerned in the points mentioned in (2) and (3). Proteins markedly deficient in the essential amino acids have long been known to be less effective than more complete ones in maintaining and repairing tissues and might presumably be similarly inefficient in controlling the appearance of abnormal lipids in periportal regions. Addition of the limiting essential amino acid to an inadequate diet might be expected to improve the performance of the protein mixture with respect to the points mentioned in (2) and (3). Many confusing and apparently contradictory observations in the literature appear capable of explanation on this basis. Experiments to test this hypothesis are now under way.

Obviously our knowledge of the factors concerned with the maintenance of normal lipids in the liver is still very incomplete. The findings reported here place further emphasis upon the importance of one factor not generally recognized as being fundamentally involved in lipotropic phenomena—i.e. an adequate supply of building blocks for the synthesis of the enzymes and lipoproteins of the liver.

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LIPIDS OF THE FEMALE REPRODUCTIVE ORGANS IN *ASCARIS LUMBRICOIDES*[1]

By Donald Fairbairn

Abstract

The female reproductive organs of *Ascaris lumbricoides* represented one fifth of the total fresh weight and contained two thirds of the total body lipids. These lipids consisted of saponifiables (79%) and unsaponifiables (21%). Phospholipids of the lecithin–cephalin type were present, as well as an abundance of triglycerides. The latter contained unusually large amounts of C_2—C_6 saturated volatile acids among which acetic and hexanoic acids predominated. Ascaryl alcohol was the major constituent of the large unsaponifiable fraction, and on the basis of previous chemical analyses and of infrared spectra a hypothetical structure is proposed. A sterol mixture containing significant proportions of both saturated and unsaturated components was also isolated, but not identified further.

Introduction

The total fats of *Ascaris lumbricoides* were first determined by Weinland (26), whose findings were confirmed by Flury (15) and von Brand (6). From results obtained in *in vitro* survival experiments (5, 6) von Brand concluded that body fats were not consumed by the female parasite for purposes of energy production, even though the earlier work of Flury had shown that a large part of the total lipids consisted of volatile and non-volatile fatty acids. Flury's data indicated clearly that much of the total fat was located in the reproductive organs, and, almost simultaneously with Fauré-Fremiet (13), he isolated from ascaris considerable quantities of a new lipid, which he named ascaryl alcohol and which was said by Fauré-Fremiet to occur only in the female reproductive organs. These authors disagreed on the nature of ascaryl alcohol, and it remained for Schulz and Becker (23) to confirm and extend Flury's results. Schulz and Becker suggested that glycerol accounted for three of the four oxygen atoms of ascaryl alcohol (empirical formula, $C_{33}H_{68}O_4$), with two of its three hydroxyl groups being free and the third substituted in ether linkage with an unidentified higher alcohol. None of these workers had succeeded in finding sterols in ascaris or in other nematodes.

Before undertaking in this laboratory a study of the fat metabolism of developing ascaris eggs (9, 12), it seemed desirable to reinvestigate the fats of the female reproductive organs, using modern analytical methods, and paying particular attention to their peculiarities, such as the presence of large amounts of ascaryl alcohol and volatile acids, and the alleged absence of sterols. The results obtained have confirmed and extended those of earlier workers, except that significant quantities of sterols have been found.

Methods

Specimens of hog ascaris were collected in warm saline solution at the slaughterhouse and maintained at 39° in the laboratory in a modified Ringer

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solution (1). Not more than 48 hr. after collection, mature females were incised longitudinally, the intestine removed and discarded, and the reproductive organs (ovaries, oviducts, and uteri) and muscle (including the cuticle) separated and blotted free of adhering perienteric fluid. The pooled reproductive organs (or muscle) were then dried overnight in a vacuum oven at 52°.

Total lipids were obtained by blending the dry tissues repeatedly with chloroform–methanol (2 : 1, v/v) and washing the combined extracts with a large excess of water (16) in order to remove non-lipid impurities. The washed solution was then evaporated to dryness and the residue dissolved in solvent, any insoluble portion being discarded.

Phospholipids were isolated from a portion of the total lipids by precipitation with acetone and ethanolic magnesium chloride solution in the usual manner. The concentration of phospholipids did not exceed 2 mgm. per ml. of final volume, for otherwise ascaryl alcohol, which is sparingly soluble in acetone, was also precipitated. The phospholipids were washed once with acetone, then dissolved in ether, washed with water, dried, and weighed. Nitrogen (25) and phosphorus (2) were determined colorimetrically.

The remainder of the lipid extract was dried and dissolved in sufficient hot ethanol to make a 15% solution. This solution was then saponified by adding a slight excess of saturated sodium hydroxide solution and heating until one half the ethanol had been distilled. Ten volumes of ether were added to the warm residue and the soaps were removed by extracting four–six times with three volumes of water. This procedure (23) was the only one which afforded a satisfactory separation of soaps from ascaryl alcohol.

The ethereal solution of unsaponifiables was dried over anhydrous sodium sulphate at room temperature, then concentrated to 1.5 volume and cooled at 2°. Precipitated ascaryl alcohol was removed by filtration and washed twice with cold ether. Evaporation of the mother liquor to 0.3 volume with subsequent cooling provided a second crop of the alcohol, which was combined with the first.

3,β-Hydroxysterols were obtained from the residue of unsaponifiables still present in the mother liquor by dissolving this residue in a minimum of ethanol and adding a 2% ethanolic solution of digitonin until precipitation of the sterol digitonides was complete. After 24 hr. the suspension was centrifuged and washed once with ether–ethanol (2 : 1) and three times with ether. Sterols were recovered from the digitonides by the method of Schoenheimer and Dam (21), and an assessment of the degree of unsaturation obtained by determining iodine number (27) and color development (22) relative to pure cholesterol. The acetates were prepared by refluxing the digitonides in acetic anhydride solution. Bromination of the unsaturated sterol and subsequent isolation of the saturated sterol component as the digitonide were carried out by an accepted procedure (20).

A portion of the alkaline aqueous solution of saponifiable substances was acidified to Congo red paper by addition of sulphuric acid, and distilled without

reflux in order to remove the volatile acids (11). After neutralization the distillate was evaporated to dryness at 110°. Volatile acids were then extracted into chloroform and fractionated on silica gel (10, 11).

Non-volatile acids remaining in the distillation flask were extracted into ether. Their neutral equivalent was determined in neutral, carbon dioxide-free ethanol solution by titration with sodium ethoxide, and iodine number (I. N.) by the Rosenmund-Kuhnhenn method (27). Solid (saturated) and liquid (unsaturated) acids were separated by a modified Twitchell reaction (24).

Results

The main features of the distribution of total lipids in *A. lumbricoides* females are summarized in Table I. Some 20% of the lipids were found in the muscle, which accounted, however, for 70% of the total dry weight of the parasite. In contrast, the reproductive organs, which comprised only 24% of total dry weight, contained 66% of the total lipids. These data are in reasonable agreement with those of Flury (15) and thus reaffirm the importance of the reproductive organs in fat metabolism. It may be mentioned that most of the reproductive tissue fat is contained in the developing oöcytes of the ovaries and in the fertilized eggs in the uteri, there being little or no depot fat or adipose tissue in the conventional sense.

Lipids obtained from the pooled reproductive organs of many females were fractionated, with results detailed in Table II. Unsaponifiable substances were abundant and accounted for more than 20% of the total. Phospholipids, although comprising a small part of the total fats, constituted a significant part (3%) of the dry, fat-free tissue. Their nitrogen (1.71%) and phosphorus (3.70%) content, and molar nitrogen to phosphorus ratio (1.04) suggested the absence of significant proportions of diaminophospholipids and confirmed indirectly certain data of Rogers and Lazarus (19) who found the phospholipids of the ovaries to consist chiefly of lecithins and cephalins. A very faint positive reaction for acetalphospholipids (plasmalogens) was noted (14).

TABLE I

DISTRIBUTION OF TOTAL LIPIDS IN THE FEMALE ASCARIS*

Tissue	Relative tissue weight		Lipids		
			% Concentration in tissue		Relative concentration
	Wet	Dry	Wet	Dry	
Whole female	100	100	1.64	8.9	100
Muscle	53	70	0.69	2.8	22
Reproductive organs	20	24	6.04	27.2	66

** Fresh weights of tissue analyzed were: whole worm, 1480 gm.; muscle, 620 gm., reproductive organs, 337 gm. The remaining tissues (perienteric fluid and intestines) were not examined.*

TABLE II

LIPIDS OF THE FEMALE REPRODUCTIVE ORGANS*

Fraction	Concentration, %wet weight	Relative concentration
Total	6.05	100
Volatile acids†	0.78	12.9
Non-volatile acids	3.14	51.9
Unsaponifiables	1.29	21.4
Phospholipids‡	0.50	8.3

* *By analysis of 711 gm. fresh weight of tissue.*
† *Calculated from titrimetric data on individual acids.*
‡ *These data must be excluded in calculating recoveries.*

Non-volatile acids were not examined in detail. Their neutral equivalent (298) was similar to that of C_{18-20} acids, and their iodine number (96) was unexpectedly high. Fractionation by the lead salt method gave 25% solid acids (I.N., 12) and 75% liquid acids (I.N., 136). The data will be more readily assessed when the results of separate analyses on triglyceride, and phospholipid acids become available.

Volatile acids (12.9% of the total lipids) were found in much smaller quantities than was expected, since Flury reported 31%, but they remain a highly significant fraction. Flury's volatile fraction presumably included free tissue acids and other volatiles, whereas the present data represent only those acids which were originally esterified. This was demonstrated by failure to find titratable or volatile substances in sodium carbonate washings of the total lipid extract. Furthermore, the total lipids were virtually odorless, but upon saponification with insufficient alkali, it was observed that ethyl esters of the volatile acids, distinguishable by their characteristic odor, were formed.

Fractionation of the volatile acids into their individual components (with respect to carbon chain length) showed that acetic, pentanoic, and hexanoic acids were predominant, with propionic and butyric acids present in small amounts (Table III). In broad outline this pattern followed that previously established for perienteric fluid by Moyle and Baldwin (17) and for excreted fermentation acids by Bueding and Yale (8), but acetic acid was much more abundant, and hexanoic acid (or acids) was present in excess of pentanoic acids. It is well to remember that the results obtained for reproductive organs are based on esterified acids alone; Moyle and Baldwin's procedure for isolating the volatile acids from perienteric fluid was such that both free and combined acids would be obtained, and the excreted fermentation acids are, presumably, free. Volatile acids of the reproductive organs, like those of the perienteric fluid, contained no unsaturated components (zero iodine number) and thus differed from excreted acids, which were shown by Bueding (7) to contain *cis*-α-methylcrotonic (tiglic) acid as a component of the C_5 fraction.

TABLE III

VOLATILE ACIDS OF THE FEMALE REPRODUCTIVE ORGANS, PERIENTERIC FLUID (17), AND EXCRETION PRODUCTS (8)

Acid	Reproductive organs*	Perienteric fluid†	Excretion products‡
	Moles per cent total volatile acids		
Acetic	39	11	15–25
Propionic	5	4	10–15
Butyric	3	2	2– 5
Pentanoic	17	46	40
Hexanoic	37	36	20

* *Glyceride acids only; 8.2 mM. per 100 gm. wet weight of tissue.*
† *Total acids; 0.38 mM. per 100 ml. fluid.*
‡ *Free acids; ca. 2.5 mM. excreted in vitro per 100 gm. ascaris in 24 hr.*

The large unsaponifiable fraction of the total lipids (Table II) contained ascaryl alcohol (82%), sterols (1.7%), and unknown substances (16%). The latter were solids at room temperature and freely soluble in ether and other solvents. A single recrystallization of the ascaryl alcohol (m.p. 79°) from chloroform and ether (90% yield) gave a product melting at 83°, in agreement with the results of Schulz and Becker (23). Infrared analysis of this product showed strong bonded hydroxyl absorption at 3400 cm.$^{-1}$ and sharp narrow band at 711 cm.$^{-1}$ identifiable with a linear chain of five or more methylene groups. Confirmation of a proposed ether linkage (23) was less certain, although there was a fairly strong absorption between 1100 and 1150 cm.$^{-1}$.

The demonstration of 3,β-hydroxysterols (m.p. 123°–125°) in ascaris was of interest in that these sterols had not previously been found in the phylum Nematoda, and it seems certain that at least two sterols, one saturated and one unsaturated, were present. Thus, from material isolated early in the investigation it was possible to prepare a few milligrams of the acetates, m.p. 109°–110° (cholesterol acetate, 114°–115°) whose infrared absorption resembled closely that of authentic cholesterol acetate, but with differences too great to be attributed to impurities. A reduced absorption at 1445 cm.$^{-1}$ suggested that this material was less unsaturated than cholesterol acetate. This interpretation was strenghened by the results of later experiments, for the color-producing capacity and iodine number of ascaris sterols were only 0.63 and 0.75, respectively, those of pure cholesterol, and the optical rotation $(\alpha)_{D^{23°}} = -12.9°$ (8.9 mgm. sterols per ml. chloroform, in a 10 cm. tube) favored the presence of considerable amounts of a saturated sterol. By bromination of the mixed sterols (20) and subsequent addition of digitonin, a saturated digitonide was isolated, which upon decomposition (21) yielded 32 mgm. of free sterol (m.p. 126°–127°, I.N. 4) equivalent to 23% of the total sterols. This product was probably impure (m.p. cholestanol, 142°) and attempts to purify it by oxidative destruction of unsaturated impurities (4) were unsuccessful.

Discussion

The lipids of ascaris reproductive organs evidently differ significantly in several ways from those of most animals hitherto studied. Among their more interesting features may be mentioned the abundance of a unique higher alcohol, the complexity of the sterols, and the existence of quantities of esterified volatile acids. The high concentration of the total fats is of interest in itself, for it points to the importance of these fats in the economy of the reproductive organs.

The structure of ascaryl alcohol is still unknown, although it was isolated over forty years ago and is readily available. From the meager evidence at hand, a hypothetical but reasonable structure would be that of a monohydrated glyceryl ether of myricyl alcohol, $CH_3(CH_2)_{28}CH_2OCH_2CHOHCH_2OH \cdot H_2O$ a structure analogous to those of the well-known batyl, chimyl, and selachyl alcohols of fish oils. The abundance of ascaryl alcohol in the reproductive organs, and its alleged confinement to them (13) suggests that it may have an important and specialized function in ascaris (and possibly in other nematodes). Fauré-Fremiet (13) claimed, as a result of histological observation, that the alcohol was formed in the cytoplasm of the developing germ cells and oöcytes, and following fertilization of the egg in the oviducts was discharged from the cytoplasm to form the extremely tough and impervious vitelline membrane. We have recently succeeded in showing that ascaryl alcohol is indeed a major component of this membrane (12).

Saturated sterols, cholestanol particularly, are well-known as minor components of vertebrate sterols (20). The much larger proportion of a saturated sterol in ascaris is comparable in some respects to the situation existing in the sponges (Porifera), and Bergmann (3) has observed that in general the sterols of primitive animals are complex.

On the other hand, the phospholipids appear to be relatively simple in nature. Rogers and Lazarus (19) found serine-, ethanolamine-, and choline-containing phospholipids in the ovaries, but little sphingomyelin, and these results are compatible with a nitrogen–phosphorus ratio approaching unity as found in the phospholipids of the entire reproductive system. Only a trace of acetalphospholipids was found, although the same test (14) applied to phospholipids isolated from ascaris muscle was strongly positive.

The presence of considerable amounts of esterified volatile acids in the reproductive organs is of interest in that such acids are rarely found in tissue fats, and it is known that similar acids are excreted by ascaris as end products of its powerful aerobic and anaerobic fermentations (8). Apparently a part at least of these energetically wasteful fermentation products can be recovered by esterification in the parasite itself; and in another communication it will be shown (9) that the esterified volatile acids are in fact utilized in the oxidative metabolism of the embryonating egg. The nature of the esterified hexanoic, pentanoic, and butyric acids is not known. In perienteric fluid Moyle and Baldwin (17) concluded that the hexanoic fraction was not normal acid, but that the C_4 fraction was *n*-butyric. They could not detect the

cis-α-methylcrotonic (tiglic) and *n*-valeric acids isolated by Bueding (7) from the excreted fermentation acids, but confirmed Bueding's isolation of α-methylbutyric acid. Our results likewise show that α-methylcrotonic acid and other unsaturated acids are not present in the esterified volatile acids. It may be that α-methylbutyric is the usual C_5 acid produced *in vivo*, and that α-methylcrotonic, its unsaturated precursor, is excreted only as a result of the exaggerated metabolism which occurs during the *in vitro* life of the surviving parasite.

Although it was not possible to determine the Reichert-Meissl number (number of ml. of 0.1 *N* NaOH required to neutralize the liquid volatile acids distilled from 5 gm. fat) of reproductive tissue fats by an official method (18), calculation from the data available (Table III) gave the value 68. This may be compared with an average value near 30 for butterfat, which is considered to be a rich source of the lower molecular weight volatile acids, and provides a useful index of the extensive incorporation of such acids into ascaris fat.

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THE PYRUVIC PHOSPHOFERASE OF ERYTHROCYTES

I. PROPERTIES OF THE ENZYME AND ITS ACTIVITY IN ERYTHROCYTES OF VARIOUS SPECIES[1]

By P. F. Solvonuk and H. B. Collier

Abstract

Mammalian erythrocytes contain a pyruvic phosphoferase (PPFase) which is activated by K^+ and Mg^{++} and inhibited by Na^+ and Ca^{++}. The K^+ can be replaced by Rb^+ or NH_4^+, and the Mg^{++} can be partially replaced by Mn^{++} or Co^{++} as activators of the enzyme. The PPFase apparently requires free thiol groups for its activity, as it is completely inhibited by 10^{-4} *M* *p*-chloromercuribenzoate and this inhibition is partially reversed by glutathione. The mean PPFase of the erythrocytes of six mammalian species was determined and found to be in the following order of decreasing activity: man, rat, dog, rabbit, cat, ox. The erythrocytes of chicks and of newborn rats showed a very high PPFase activity.

Introduction

Activation of a specific enzyme system by K^+ was first demonstrated by Boyer, Lardy, and Phillips (4) with pyruvic phosphoferase (PPFase). This enzyme catalyzes the transfer of phosphate from phospho(enol)pyruvic acid (PEPA) to adenosine diphosphate (ADP) with the formation of free pyruvate and adenosine triphosphate (ATP). The PPFase thus plays an important role in the cellular glycolytic mechanism. (Hoffmann-Ostenhof (8) prefers to call the enzyme "phosphopyruvic–ADP transphosphatase" but we shall retain Boyer's term until the terminology of the transferring enzymes has been standardized.)

Kachmar and Boyer (9) demonstrated the indispensability of the cations K^+, Rb^+, or NH_4^+ for PPFase, and recently Boyer (2) determined the activity of the enzyme in 19 different tissues of 11 vertebrate and invertebrate species. However, he did not examine the formed elements of the blood.

As one aspect of an investigation of the cation gradient of the erythrocyte, we have reported (6) the occurrence of PPFase in the red cells of six mammalian species and its activation by K^+. The present paper provides further information on the properties of this enzyme.

Materials and Methods

PPFase activity was determined by a modification of the method of Kachmar and Boyer (9). Blood was collected in heparinized tubes, the erythrocytes were washed three times with 0.15 *M* potassium chloride, and the buffy layer was removed by aspiration at each washing. (Bartlett and Marlow (1) have recently estimated the contribution of the leukocytes to glycolysis of whole blood, and we have concluded from their data that the few remaining leukocytes in our washed cell suspensions could contribute but little to the total activity.) The packed cells were suspended in nine times

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their volume of the potassium chloride solution and aliquots were removed for cell counts and for PPFase determinations. For human erythrocytes, 1.0 ml. of the 1 : 10 suspension was centrifuged and the supernatant was removed by aspiration. The cells were then hemolyzed with 5.0 ml. of water and 0.1 ml. of the hemolyzate was used for each enzyme determination.

Phospho(enol)pyruvic acid was obtained as the silver, barium salt (California Foundation for Biochemical Research) and the heavy metals were removed by treatment with hydrochloric acid and potassium sulphate. ADP was used as the sodium salt (Sigma Chemical Co.); it was found necessary to prepare fresh solutions daily. TRIS buffer, pH 7.4, was prepared from tris(hydroxymethyl)aminomethane and hydrochloric acid.

The incubation mixture was made up to the following final molar concentrations: PEPA, 0.003; ADP, 0.004; TRIS buffer, 0.040; KCl, 0.10; $MgSO_4$, 0.005. A 0.90 ml. volume of this solution was brought to the water-bath temperature of 37° C., 0.10 ml. of hemolyzate was added, and the mixture was incubated for exactly 10 min. ADP was omitted from the controls. At the end of the incubation period, free pyruvate in the whole mixture was determined by the dinitrophenylhydrazine method (9). Crystalline sodium pyruvate was used as the standard for the photometric readings in the Beckman DU spectrophotometer at 510 mμ. Absorbancy readings were proportional to pyruvate concentration up to about 1 μ M. per ml. of incubation mixture, or 5 ml. of final solution as read in the photometer.

PPFase activity was expressed as micromoles of pyruvate liberated in 10 min. at 37° C. in 1.0 ml. of incubation mixture. Under these conditions the liberation of pyruvate, in the range 0–0.6 μ M./ml., varied directly with enzyme concentration. For comparison of the mean erythrocyte PPFase of various species, the activity was expressed as micromoles of pyruvate liberated per 10^9 cells.

Results

Preliminary experiments indicated that the erythrocyte 'ghosts' were devoid of PPFase activity, and that the enzyme is therefore intracellular; accordingly, whole hemolyzates were used in subsequent experiments. Tests were made

TABLE I

PHOSPHOFERASE ACTIVITY OF HUMAN BLOOD STORED IN ACID–CITRATE–DEXTROSE AT 5° C.

Storage period, days	Micromoles pyruvate/10^9 cells
0	8.2
2	8.0
5	7.6
8	7.4
11	7.4
15	6.3
21	4.7
29	2.1

of the stability of the enzyme. An acetone powder of red cells showed considerable activity, and the figures in Table I indicate that whole human blood loses little of its activity when stored in the refrigerator in acid–citrate–dextrose for a few days. (The decrease in activity on storage cannot be attributed to loss of K^+ and gain of Na^+ by the erythrocytes, since the dilute hemolyzates are brought up to optimum K^+ concentration, and there is too little Na^+ to cause inhibition.) Hence the enzyme may be regarded as quite stable. Nevertheless fresh blood was always used in the present experiments.

Effect of Various Cations

Prior to the testing of the cation requirement of the enzyme, hemolyzates of human cells were dialyzed in the refrigerator against TRIS buffer, pH 7.4. It was found that 12–30 hr. dialysis resulted in complete loss of activity, even when optimum amounts of K^+ and Mg^{++} were restored. (The optimum concentration of K^+ was in the range 0.05–0.15 *M* while that of Mg^{++} was 0.005–0.010 *M*.) In contrast to this, the muscle PPFase of Kachmar and Boyer (9) seemed quite stable to dialysis. The activity of our dialyzates was not restored by addition of glutathione, cysteine, boiled hemolyzate, or boiled yeast extract; but addition of 2% crystalline plasma albumin (Armour) to the hemolyzates before dialysis was effective in preventing inactivation.

Table II records the effect of univalent cations upon dialyzed hemolyzates of human cells. Mg^{++} was added to a final concentration of 0.005 *M* in every case. The PPFase activity is completely lost in the absence of K^+; this ion can be replaced by Rb^+ or NH_4^+, but not by Li^+ or the acetylcholine cation. Na^+ is inhibitory in the presence of optimum concentrations of K^+ and Mg^{++}. The observations on the effects of the inorganic cations are in accord with those of Kachmar and Boyer (9) for muscle enzyme.

The effect of adding various divalent cations to undialyzed hemolyzate of human cells is shown in Table III, where 0.1 *M* K^+ was present in all cases. In the presence of optimum concentrations of Mg^{++} all the ions are inhibitory;

TABLE II

EFFECT OF UNIVALENT CATIONS ON PHOSPHOFERASE

(Dialyzed hemolyzate, K^+ = 0.10 *M*, Mg^{++} = 0.005 *M*)

Ion	Conc., *M*	PPFase activity, % of maximum	
		In presence of K^+ and Mg^{++}	In absence of K^+
Na^+	0.001	100	
	0.10	84	
Li^+	0.10		0
Rb^+	0.10		87
NH_4^+	0.10		88
ACh^+	0.10		0

but in the absence of Mg^{++} either Mn^{++} or Co^{++} can restore partial activity. The heavy metal ions are powerful inhibitors. Kachmar and Boyer (9) showed that the inhibition of muscle PPFase by Ca^{++} could be partially reversed by increasing the concentration of K^+. We obtained similar results with the red cell enzyme, as noted in Table IV.

TABLE III

EFFECT OF DIVALENT CATIONS ON PHOSPHOFERASE

(Undialyzed hemolyzate, $K^+ = 0.10\ M$, $Mg^{++} = 0.005\ M$)

Ion	Conc., *M*	PPFase activity, % of maximum	
		In presence of K^+ and Mg^{++}	In absence of Mg^{++}
Ca^{++}	0.0001	100	
	0.001	70	
	0.010	14	
	0.10	0	
Mn^{++}	0.005	47	48
Ni^{++}	0.005	13	0
Co^{++}	0.005	24	24
Cu^{++}	0.00001	90	
	0.0001	20	
	0.001	0	
Zn^{++}	0.0001	26	
	0.001	0	0
Sr^{++}	0.005	51	0
Hg^{++}	0.00001	40	
	0.0001	6	
Pb^{++}	0.0001	70	

TABLE IV

EFFECT OF K^+ ON Ca^{++} INHIBITION OF PHOSPHOFERASE

($Mg^{++} = 0.005M$)

K^+ conc., *M*	Ca^{++} conc., *M*	PPFase, μ M./ml.	Inhibition by Ca^{++}, %
0.10	0	0.66	
0.10	0.001	0.41	38
0.20	0	0.55	
0.20	0.001	0.37	33
0.40	0	0.32	
0.40	0.001	0.25	22

Effect of Enzyme Inhibitors

Various enzyme inhibitors were added to undialyzed hemolyzates of human cells, but as shown in Table V *p*-chloromercuribenzoate (PCMB) was the only compound that exerted a significant inhibition. It was repeatedly observed that no inhibition was produced by PCMB at 10^{-5} *M*, whereas a concentration of 10^{-4} *M* always gave complete inhibition. This inhibition took effect within less than one minute after mixing the enzyme and PCMB. The affinity of PCMB for the enzyme must be quite high, as 0.1 *M* glutathione (GSH) produced only partial reactivation. Table VI illustrates the effect of adding GSH following the PCMB. It was also found that 0.01 *M* GSH gave complete protection against inhibition by 0.0001 *M* Cu^{++}, and 0.001 *M* cysteine protected against the action of 0.0001 *M* Zn^{++}. It is apparent that the enzyme requires free thiol groups for its activity.

TABLE V

EFFECT OF ENZYME INHIBITORS UPON PHOSPHOFERASE

Inhibitor	Conc., *M*	Time prior to assay, hr.	Inhibition, %
PCMB	0.000001	0	0
	0.00001	0	0
	0.0001	0	100
	0.001	0	100
Potassium ferricyanide	0.001	0	0
	0.001	1	0
	0.001	6	13
Sodium iodoacetate	0.001	0	0
	0.001	20	28
Phenothiazine	0.001	1	0
Phenothiazone	0.00003	0	9
2,4-Dinitrophenol	0.001	0	9
Alloxan	0.005	0	0
Sodium fluoride	0.010	0	0
Sodium arsenate	0.016	0	6
Urethane	0.010	0	0

TABLE VI

REVERSAL OF PCMB INHIBITION BY GLUTATHIONE

(0.0001 *M* PCMB + 0.1 *M* GSH)

Time in contact with inhibitor, min.	Time in contact with GSH, min.	Reactivation, %
1	10	18
1	15	47
1	25	59
10	25	28

PPFase of Erythrocytes of Various Species

Fresh blood specimens were used to determine the erythrocyte PPFase activity of various species of mammals. The results are summarized in Table VII where mean PPFase activity is expressed as micromoles of pyruvate liberated by 10^9 cells in 10 min. at 37°. The activity in micromoles per milliliter of packed cells has also been estimated.

The erythrocytes of the dog, cat, and ox are of the low potassium – high sodium type (12) and these in general were found to have the lower PPFase activity, whether it is calculated per cell or per unit volume of cells. It should be noted that these determinations were carried out at concentrations of $K^+ = 0.10\ M$ and $Mg^{++} = 0.005\ M$, which may not be the optimum concentration for the PPFase of all species. The determination of the optimum ionic concentration for these various types of red cells is under investigation at the present time. Further information is required before it will be profitable to speculate as to a relationship between the low enzyme activity and the low K^+ concentration of these cells.

TABLE VII

ERYTHROCYTE PHOSPHOFERASE OF MAMMALIAN SPECIES

Values are given as the mean for each species ± the standard error of the mean. Activity per ml. of packed cells has been estimated from published values for the mean cell volume*

Species	No. of animals	Mean cell volume*, μ^3	PPFase activity		
			μ M./10^9 cells		μ M./ml., estimated
			Range	Mean ± S.E.	
Man	16	87	8.0 – 15.2	11.3 ± 0.52	130
Rat	18	61	4.0 – 10.6	7.2 ± 0.51	118
Dog	10	66	4.1 – 11.0	5.9 ± 0.70	89
Rabbit	9	61	3.3 – 7.4	4.8 ± 0.50	79
Cat	8	57	1.9 – 4.7	3.4 ± 0.32	60
Ox	7	48	1.4 – 2.8	2.1 ± 0.17	44

* *Taken from "Standard values in blood", edited by E. C. Albritton. U.S. Air Force, Technical Report No. 6039, Dayton, Ohio, 1951.*

The PPFase activity of the erythrocytes of fetal and of newborn rats was measured on pooled blood from all the rats in a litter. Reticulocyte counts were made on blood smears stained with New Methylene Blue, Color Index No. 927, by the method of Brecher (5). The counts are expressed as percentages of the total red cells. As noted in Table VIII the PPFase activity of the red cells of the fetal and newborn rats is extremely high and parallels the proportion of reticulocytes. However, enzyme activity is not necessarily correlated with the reticulocyte count in all cases, as will be discussed in the second paper of this series, on the PPFase activity of erythrocytes of experimental animals and human patients (14).

Table VIII also includes measurements on human cord blood which show that the mean PPFase activity of the erythrocytes is only slightly higher than the mean value for adult cells.

Values for the nucleated erythrocytes of the chick, as included in Table VIII, are much higher than those listed for the mammals. Hemolyzates of chick cells had the same PPFase activity before and after centrifugation, which indicates that the enzyme is not associated with the nucleus. Avian erythrocytes, under aerobic conditions, have a high rate of respiration and low glycolytic activity, as compared with mammalian red cells; while anaerobically the glycolytic rate is of the same order of magnitude as that of the mammalian cells (13). It is not clear why these nucleated cells should exhibit such a high PPFase activity.

TABLE VIII

MEAN PHOSPHOFERASE OF IMMATURE AND OF NUCLEATED ERYTHROCYTES

Species	Age	No. of animals	PPFase, μ M./10^9 cells		Mean retic. count, %
			Range	Mean ± S.E.	
Rat*	Fetal	1		95	100
	1 day	3	88 – 124	102	98
	3 days	1		110	78
Man	Cord blood	10	10.0 – 21.3	17.1 ± 1.67	4
Chick	1 month	5	75 – 107	87 ± 5.2	

** The blood from 5–8 young rats of the same litter was pooled for the PPFase determination. The figures in the third column represent the number of such pools analyzed.*

Discussion

Our results indicate that the erythrocyte PPFase is identical with that of muscle, except that it appears to be more labile to dialysis. The protective action of plasma albumin is presumably a non-specific effect, and the muscle extracts probably contain protein that exerts a similar action. The effects of the univalent cations on the red cell enzyme, and the inhibitory effect of Ca^{++} and its partial reversal by K^+, are similar to those reported for the muscle enzyme. All the divalent cations that we tested were inhibitory in the presence of K^+ and Mg^{++}, but Mn^{++} and Co^{++} could partially replace Mg^{++} as activators. Probably their inhibition of the enzyme in the presence of Mg^{++} is of a competitive nature. It is interesting to note that phosphoglucomutase of rabbit muscle is activated by Mn^{++}, Ni^{++}, and Co^{++} as well as by Mg^{++} (7).

In view of the striking antagonism between K^+ and Na^+ and between Mg^{++} and Ca^{++}, this enzyme system seems to be eminently suitable for a further study of these ion antagonisms which are of such fundamental physiological

importance. The mechanism of such antagonisms may be explained by the studies of Melchior (10) on the configuration of the ATP molecule and the modifications in this configuration that are induced by the binding of cations with various ionic radii. Boyer and Harrison (3) have also discussed the role of K^+ and Mg^{++} in this enzyme system.

The powerful inhibition of PPFase by *p*-chloromercuribenzoate and its partial reversal by GSH indicate that the enzyme requires free thiol groups for its activity, as was reported by D. M. Needham in 1942 (11).

It is interesting to note that, of the mammalian species examined, the erythrocytes of the low potassium – high sodium type tend to have a lower cellular PPFase activity. However, the mechanism of potassium accumulation in red cells has not been elucidated, and there is no evidence of any causal relationship between cellular K^+ concentration and PPFase activity. The high PPFase activity of nucleated and immature erythrocytes parallels their high metabolic activity, but the significance of this relationship is not clear at the present time.

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ON RAT SERUM AMYLASE

II. THE INFLUENCE OF DIET ON LEVELS OF THE ENZYME[1]

By G. Stuart Wiberg and Jules Tuba

Abstract

The relationship between food consumption and rat serum amylase previously reported has been further investigated, this time with the use of synthetic diets. With high carbohydrate diets neither sucrose nor corn starch exerted any significant influence on the rat serum amylase level. A high concentration of protein in the diet produced a highly significant decrease in the activity of the enzyme. On the other hand, a high fat diet resulted in a highly significant elevation of serum amylase. The effect of fat was proportional to concentration up to a limiting value. In diets which lacked carbohydrate or when the carbohydrate was completely or largely indigestible, levels of the enzyme were depressed significantly below normal. With protein-free diets, a regimen consisting largely of fat and carbohydrate, enzyme levels were depressed significantly when sucrose was used, whereas amylase values were normal when starch was used as the carbohydrate.

Introduction

The effect of diet on serum amylase has been the subject of extensive investigation. Most workers have concluded that diet has no influence (3, 4, 5, 6, 7, 8, 9, 10, 14, 16, 17, 20, 23), although a few have stated that certain diets could modify the levels of serum amylase (2, 12, 21, 22, 24). This discrepancy in results is associated at least in part with the lack of standardized experimental conditions. For example, human serum amylase levels were determined when the subject was in the fasting state, and then one to three hours after the ingestion of a test meal identified only as high in concentration of carbohydrate or fat or protein (9, 10, 19, 20). It has also been reported that fasting is without effect on serum amylase, although here the period of food deprivation was limited to 24 hr. generally (3, 9, 10, 20). However, it has been shown by Flock and Bollman (11), Tuba and Wiberg (29), and Wiberg, Little, and Tuba (31), that fasting produced very pronounced decreases in levels of the enzyme in rats, but three days without food were required to lower the values to a degree highly significant (29).

Enzymic adaptation by bacteria in response to alterations in nutritional environment has been a well-recognized phenomenon since the work of Wortmann in 1882 (32). It is essential before such changes become fully manifested that the organism, either bacterial or animal, be given sufficient time to adjust itself to the altered nutritional *milieu*. For example, Grossman, Greengard, and Ivy (13) found that diets high in carbohydrate or protein produced marked changes in some of the pancreatic enzymes of the rat in 21 days. Previous work in this laboratory has indicated that both alkaline phosphatase and lipase of rat serum respond to changes in the nature of the

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host's diet (25, 26, 27, 28), and that the amount and nature of the dietary fat was the most important factor affecting the response. Squires (22) in a study of certain native African tribes found that there was a parallelism between salivary amylase activity and established levels of carbohydrate consumption. He also noted a significant correlation between the levels of the enzyme in serum and in saliva (21).

It appeared to us from a survey of previous work and from our own experience in this laboratory, that in any attempt to study the influence of various dietary factors on rat serum amylase, rigorous controls should be maintained. These should include: the age and weight of the animals, diets of uniform composition and purity, and a continuation of the dietary regimens for a period of time sufficient to allow for enzymic adaptation.

Experimental and Results

Adult male rats of uniform age and weight within each group were utilized. The animals were housed in individual cages and allowed ground Purina fox checkers (to permit accurate estimation of consumption) and water ad libitum for at least a week. They were then fasted for three days (30) and placed on the experimental dietary regimen for periods of at least 28 days. With each series of experiments a group of animals was maintained throughout on ground Purina fox checkers as a further control.

Serum amylase determinations were made at weekly intervals according to the procedure previously outlined (29). The unit of amylase activity is that amount of the enzyme sufficient to hydrolyze 1 gm. of starch in one hour at 27° C. at pH 7.0. Not more than 50% of the substrate should be hydrolyzed during the one hour digestion period. Results are expressed in units of amylase activity per 100 ml. serum. Body weights were recorded at the same time as blood was collected. Daily records of food consumption were also maintained.

Other than the control regimen of ground checkers, each diet was synthetic. These synthetic diets contained fat in the form of Crisco, casein, and either starch or sucrose, in the proportions indicated below, as well as 4% McCollum's salt mixture (18). Each diet was supplemented with 5 mgm. thiamine hydrochloride, 5 mgm. pyridoxine hydrochloride, 5 mgm. nicotinamide, 20 mgm. calcium pantothenate, 10 mgm. riboflavin, 1 gm. choline chloride, 1.2 mgm. vitamin A acetate, 0.14 mgm. calciferol, and 120 mgm. α-tocopherol per kilogram of diet. Because thiamine has been found to be unstable in synthetic diets (15), each diet was supplemented with 500 mgm. ascorbic acid per kilogram of diet to act as an antioxidant.

High Sucrose, High Starch, High Protein, and High Fat Diets

The first two diets contained sucrose or corn starch to the extent of 70%, and in addition 18% casein, 8% fat, and the minerals and vitamins as indicated above. The high protein diet contained 66% casein, 22% sucrose, and 8% fat. The fourth diet, high in fat, contained 45% Crisco, 33% sucrose, and

TABLE I

EFFECT OF HIGH CARBOHYDRATE, HIGH PROTEIN, AND HIGH FAT DIETS ON RAT SERUM AMYLASE (A) IN UNITS PER 100 ML., BODY WEIGHT (W) IN GM., AND FOOD CONSUMPTION (C) IN GM. PER DAY

(Means of six rats)

Time	Control			High sucrose			High starch			High protein			High fat		
	A	W	C	A	W	C	A	W	C	A	W	C	A	W	C
Prediet	55.4	382	22	58.3	295	21	61.9	297	20	60.4	320	21	57.4	303	21
Diet															
Week 1	61.0	390	21	61.8	300	16	66.7	301	19	49.9	326	15	72.5	328	15
Week 2	59.3	397	22	58.2	311	15	61.4	312	16	46.3	336	15	67.2	349	13
Week 3	57.9	403	20	56.8	318	14	60.5	323	16	45.1	345	14	67.9	365	12
Week 4	58.9	404	21	57.0	326	14	59.3	331	16	49.8	350	12	67.6	378	12
Postdiet															
Week 1	58.0	412	20	58.9	335	16	61.0	347	17	61.9	355	19	59.9	380	14
Week 2	63.2	413	23	59.2	339	23	60.5	346	20	64.2	366	22	59.1	380	19

Standard errors of means are not included because of the size of the tables. The value of t is not included in the text in statistical analysis since this might involve several values per experiment. However, the values of P are given to indicate levels of significance.

18% casein. The predietary values for serum amylase, body weights, and daily food consumption are given in Table I. In addition, these values are indicated at weekly intervals during the four-week ad libitum experimental feeding period, as well as for two weeks after the animals were restored to the control diet of ground checkers.

One might have expected that the high starch diet would have resulted in some significant alteration of amylase levels. However, although the serum amylase tends to be higher with the high starch diet than with the high sucrose diet, in neither case are levels significantly altered from the control values. Consumption values for the two high carbohydrate diets are markedly less than for the control diets, but not to a sufficient extent to affect enzyme values through inanition (29). The high protein diet in four weeks effected a highly significant diminution in rat serum amylase levels ($P < 0.01$). Following return to the control diet, increased consumption was accompanied by elevation of enzyme values to normal. The results for the high fat diet were surprising and the highly significant increase in enzyme activity ($P < 0.01$) was quite unexpected. Our findings are strengthened by the fact that the above experiment, with diets high in carbohydrate or fat or protein, was repeated twice and the results do not differ materially from those in Table I.

We have completed studies on the amylase content of various tissues of rats which had subsisted on the dietary regimens discussed above. The results of these studies, which are not included in this paper, are in substantial agreement with those of Grossman *et al.* (13) concerning pancreatic amylase, and we shall show in a subsequent publication that they provide a rationale for some of the results of Table I.

Diets with Varying Concentrations of Fat

Because fat had such a highly significant effect on levels of rat serum amylase, it was decided to investigate the effect of fat at different dietary concentrations to see if the degree of response by the enzyme was proportional to the fat concentration. Accordingly a series of diets was prepared, which contained fat at levels of 0, 5, 15, 25, 45, and 60%. All these diets contained 18% casein, 4% salts, and the usual supplements of vitamins. The balance of the diet in each case was made up with sucrose.

It will be noted in Table II that the fat-free diet resulted in the lowest amylase levels. There was a gradual increase in enzyme activity as the dietary fat concentration was raised from 0 to 45%. After four weeks the group of rats subsisting on the 60% fat diet manifested amylase values below those for the group which were fed the 45% Crisco diet. This highest concentration of dietary fat resulted in a generally lower food consumption over the experimental period and so other metabolic and physiological factors became operative. For example, over the four week experimental period the group of rats maintained on the 45% Crisco diet showed a mean weight gain of 75 gm., while the group subsisting on the 60% fat diet gained but 44 gm.

TABLE II

THE EFFECT OF DIETARY FAT LEVEL ON RAT SERUM AMYLASE (A) IN UNITS PER 100 ML., BODY WEIGHT (W) IN GM., AND FOOD CONSUMPTION (C) IN GM. PER DAY

(Means of six rats)

Time	Control			0% fat			5% fat			15% fat			25% fat			45% fat			60% fat		
	A	W	C	A	W	C	A	W	C	A	W	C	A	W	C	A	W	C	A	W	C
Prediet	62.2	345	21	57.7	340	22	56.8	355	23	57.5	363	21	54.7	320	21	57.4	303	21	51.2	346	20
Diet																					
1 week	66.4	362	21	55.7	348	15	57.8	360	17	59.8	382	16	63.9	353	12	72.5	328	15	63.8	360	11
2 weeks	62.8	377	20	54.1	359	17	58.4	374	16	61.6	392	13	61.4	358	14	67.2	349	13	59.5	369	10
3 weeks	62.7	372	21	60.0	362	14	59.4	377	16	63.6	396	12	64.5	367	11	67.9	365	10	64.2	379	10
4 weeks	66.9	391	20	54.4	374	14	59.7	390	14	62.9	408	14	63.3	378	14	67.6	378	12	59.8	390	12
Postdiet																					
1 week	57.7	401	21	59.7	382	22	57.6	395	22	58.3	412	18	60.6	384	19	59.2	380	19	57.6	388	16

Statistical treatment of the data in Table II shows a highly significant correlation between the amount of fat in the diet and the level of the serum enzyme ($r = 0.989$, $t = 3.56$, $P < 0.01$).

Diets Lacking in Available Carbohydrate

A group of animals was maintained for the usual period of four weeks on a synthetic diet which contained no carbohydrate. This regimen consisted of the usual vitamins and salts, 11% fat and 85% protein, so it was, in effect, a carbohydrate-free, high protein diet. Two other diets were used in this experiment, in which the usual readily digestible carbohydrate (starch or sucrose) was replaced by the same amount (70%) of cellulose (not digestible) or uncooked potato starch (not readily digestible (1)).

TABLE III

THE EFFECT OF DIETS FREE OF AVAILABLE CARBOHYDRATE ON RAT SERUM AMYLASE (A) IN UNITS PER 100 ML., BODY WEIGHT (W) IN GM., AND DAILY FOOD CONSUMPTION (C) IN GM.

(Means of six rats)

Time	Control			CHO free			Cellulose			Raw potato starch		
	A	W	C	A	W	C	A	W	C	A	W	C
Prediet	60.2	373	21	61.8	328	21	63.7	334	19	58.3	341	20
Diet												
Week 1	61.0	377	21	61.8	329	14	60.2	332	18	59.2	335	15
Week 2	59.4	379	20	51.7	333	16	42.5	297	25	49.5	337	23
Week 3	59.3	389	22	52.9	324	13	36.1	281	29	46.0	339	20
Week 4	57.9	384	21	49.4	342	12	36.1	273	31	44.5	343	17
Postdiet	58.9	383	21	58.5	353	18	58.4	308	24	64.7	373	24

The data for rat serum amylase presented in Table III show that the three experimental diets had produced in one week no effect that is significantly different from the pre-experimental or control values. However, by the end of the second week amylase levels had fallen in the three experimental groups to levels which are highly significant ($P < 0.01$), and which were lowest in the cellulose-containing diet. The cellulose diet also produced loss of weight and an abnormally great consumption of food. The data for this group listed in the table are similar to those noted by us previously with fasted animals (29, 31). On both the carbohydrate-free and raw potato starch diet good growth was maintained. The lack of readily digestible carbohydrate in the latter regimen seems to be the factor associated with the lowered amylase levels. The enzyme values for the group which received no carbohydrate were influenced by the high protein nature of the diet (Table I) as well as the abnormally low consumption; and it would be useless to speculate about what effect, if any, may be ascribed to the absence of carbohydrate.

Protein-free Diets

Two protein-free diets were finally studied. Both diets contained 11% fat and 85% of either sucrose or corn starch. These, as well as the usual control diet of ground checkers, were fed to three groups of six rats each, and the usual experimental procedure was followed.

TABLE IV

THE EFFECT OF THE NATURE OF THE CARBOHYDRATE IN PROTEIN FREE DIETS ON RAT SERUM AMYLASE (A) IN UNITS PER 100 ML., BODY WEIGHT (W) IN GM., AND DAILY FOOD CONSUMPTION (C) IN GM.

(Means of six rats)

Time	Control			Sucrose + Crisco			Starch + Crisco		
	A	W	C	A	W	C	A	W	C
Prediet	55.5	332	22	55.9	339	16	54.0	343	21
Diet									
Week 1	54.3	347	20	54.9	313	15	63.8	317	13
Week 2	57.1	354	23	49.9	294	11	54.4	292	13
Week 3	53.6	361	20	46.9	277	11	54.7	273	11
Week 4	57.5	361	21	46.4	260	13	55.6	257	9
Week 5	59.3	364	20	47.2	245	12	55.8	239	9
Week 6	57.9	375	21	44.1	233	11	55.6	228	9
Postdiet									
Week 1	54.9	379	22	61.1	299	27	52.4	295	20
Week 2	52.9	383	19	52.3	338	22	52.7	328	21

Some extremely interesting results are reported in Table IV. While the amylase values for the starch–Crisco diet more or less parallel those for the control group, there is a very marked and highly significant decrease in enzyme levels of the group on the sucrose–Crisco diet by the end of the second week. Since, in the latter diet, protein is lacking and since we noted above (Table II) that this level of fat (11%) does not alter serum amylase levels from the normal values, it would appear that the polysaccharide, corn starch, is one factor which stimulates the maintenance of "normal" serum amylase activity.

From these studies it is apparent that the composition of the diet has a definite influence on the level of serum amylase in the rat. This enzyme is not restricted to any one tissue or organ, and consequently its levels in the blood may be the summation of the contributions from a number of sources. At least some of the tissues may be expected to vary in the production of amylase, in accordance with certain alterations in the nutritional environment of the animals. Hence to evaluate fully the effect of diet on serum amylase activity, it has been considered necessary to study the relationship between alterations in the regimens supplied to the rats and variations of levels of the enzyme in certain tissues. This is the subject of a subsequent report.

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PHOSPHORUS METABOLISM OF THE LIVER

EFFECT OF HYPOPHYSECTOMY, ADRENALECTOMY, AND ADMINISTRATION OF ACTH ON THE INCORPORATION OF RADIOACTIVE PHOSPHATE INTO RNA NUCLEOTIDES[1]

By J. E. Logan, F. C. Heagy[2], and R. J. Rossiter

Abstract

The specific activity of the liver RNA nucleotide phosphorus, relative to the specific activity of the liver inorganic phosphate, was determined in the rat, 16 hr. after an intraperitoneal injection of radioactive inorganic phosphate. The nucleotides were isolated by ionophoresis on paper strips.

Hypophysectomy caused a decrease in the relative specific activity of each of the four RNA nucleotides. The administration of ACTH caused an increase in the incorporation of P^{32} into each of the RNA nucleotides of the liver of hypophysectomized animals, but it caused a small and statistically significant decrease in normal animals. Adrenalectomy, either in normal or in hypophysectomized rats, did not affect the P^{32} incorporation, nor did the administration of ACTH in the absence of the adrenal glands.

It is concluded that ACTH can affect the incorporation of P^{32} into the RNA of the liver and that this effect is due to the action of the hormone on the adrenal cortex. However, other factors also must be operative, since removal of the adrenal glands does not cause the decrease in the P^{32} incorporation observed after removal of the pituitary.

Introduction

Using the extraction procedure of Schneider (19) for total nucleic acid, Fraenkel-Conrat and Li (10) reported that hypophysectomy was associated with a decreased incorporation of P^{32} into the mixed nucleic acids of the liver of the rat. However, as Davidson, Frazer, and Hutchison (3) and others have pointed out, such an extraction method is not well suited to the measurement of radioisotope incorporation into nucleic acids, because of the presence in the extracts of non-nucleotide phosphorus-containing compounds of high radioactivity. Using the method of Hammarsten (13), Riedel and DeLuca (18) showed that there was a decrease in the incorporation of P^{32} into the ribonucleic acid (RNA) of the liver of hypophysectomized rats. Having regard for the importance of RNA in cellular function, more data were clearly needed on the effect of hypophysectomy and the administration of ACTH on the incorporation of P^{32} into the nucleic acids of liver.

In the present experiments, the individual nucleotides of liver RNA were separated by means of ionophoresis on paper, using the method of Davidson and Smellie (4). Since ACTH was found to affect the P^{32} incorporation in both normal and hypophysectomized animals, a further series of experiments was carried out using adrenalectomized rats and adrenalectomized rats that had previously been hypophysectomized.

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Methods

Animals

The animals were male rats of the Sprague-Dawley strain. Hypophysectomized rats, supplied by the Hormone Assay Laboratories, Inc., Chicago, Ill., were used for the experiments either two (Table I) or three (Table II) days after the operation. The adrenal glands of hypophysectomized animals displayed the typical atrophy associated with hypophysectomy. Following hypophysectomy the liver weights also were significantly lower than those of the control group. Macroscopic and microscopic examination of the pituitary region of selected animals showed no evidence of pituitary tissue remaining. Adrenalectomy was performed according to the procedure described by Farris and Griffith (9). The animals were used for experiment on the day after the adrenalectomy. In the hypophysectomy–adrenalectomy group, the adrenals were removed two days after the pituitary and the animals were taken for the experiment on the following day, i.e. three days after the hypophysectomy. Examination at autopsy indicated complete removal of adrenal tissue in adrenalectomized animals. The animals were fasted 12 to 14 hr. before killing. Control animals were carried along with each group of test animals.

ACTH Preparation

The ACTH used was obtained from Connaught Medical Research Laboratories, Toronto, Ont. It was a white powder, completely soluble in isotonic saline. The preparation had been standardized in terms of the International Standard.

Experimental Procedure

Test animals received a single intraperitoneal injection of ACTH, 4 mgm. per 100 gm. body weight, in isotonic saline. Control animals received an equivalent volume of isotonic saline. Four hours later all the animals received 200 μc. P^{32}*, regardless of body weight, by intraperitoneal injection.

The animals were anesthetized with nembutal 16 hr. after the P^{32} injection, i.e. 20 hr. after the injection of ACTH or saline. The liver was removed as rapidly as possible, blotted to remove excess blood, trimmed carefully to remove adhering adipose and connective tissue, weighed, and frozen rapidly by being plunged into liquid nitrogen.

Analytical and Counting Methods

The frozen livers were broken up into small fragments with a mortar and pestle. Approximately 300 mgm. tissue was homogenized in ice-cold 10% trichloroacetic acid using an homogenizer of the Potter–Elvehjem type. The concentration and the specific activity of the inorganic P of the trichloroacetic acid extracts were then determined by the method of Ernster, Zetterström, and Lindberg (8). The tissue residue was washed four times with 10%

* *Supplied in the form of* $H_3P^{32}O_4$ *by Commercial Products Division, Atomic Energy of Canada, Limited.*

trichloroacetic acid, in the cold, to remove the last traces of acid-soluble radioactivity. The residue was then extracted successively with ethanol (two portions), ethanol–ether 3 : 1 (five portions heated to boiling), and ether to remove lipid phosphorus. The residue remaining after removal of the lipid P was extracted three times with 10% sodium chloride at 100° C. to obtain the nucleic acids. The nucleates were precipitated with two volumes absolute ethanol, washed twice with 5% trichloroacetic acid, and dried with ethanol and ether. Hydrolysis of the nucleic acids was carried out in 0.3 *N* potassium hydroxide at 37° C. and the resulting acid-soluble RNA nucleotides were separated by ionophoresis on paper according to the method of Davidson and Smellie (4). After elution, the nucleotides were ashed with 60% perchloric acid by the method of King (15), and the concentration and the specific activity of the phosphorus determined as described for the inorganic P.

In the method of Ernster *et al.* (8), both the concentration and the radioactivity of the inorganic P are determined on the same sample. The optical density of the blue color complex was read with a Beckman Model B spectrophotometer at 730 mμ and the radioactivity due to the P^{32} in the samples was determined on the same colored solution using an M-6 liquid counter (20th Century Electronics). The counting rate was corrected for background and decay. The specific activity of each nucleotide was expressed as a percentage of that of the liver inorganic P (relative specific activity).

Results

Table I shows the effect of hypophysectomy and the administration of ACTH on the specific activity of the phosphorus of each of the RNA nucleotides relative to that of the inorganic phosphate of the liver. In the normal animals the specific activities of the adenylic acid and uridylic acid were greater than those of the guanylic acid and cytidylic acid, in agreement with the observations of Volkin and Carter (21) and Davidson and Smellie (5) for rat liver. It is noteworthy that adenylic and uridylic acids also had a higher specific activity in the adrenal glands of the rat *in vivo* (16) and the brain of the cat *in vitro* (6).

Following hypophysectomy, the relative specific activity of each of the four RNA nucleotides was considerably decreased. The administration of ACTH increased the incorporation of P^{32} into the RNA nucleotides of the hypophysectomized rats, but decreased the incorporation in normal animals. As a result there were no significant differences between normal and hypophysectomized animals after the injection of ACTH.

To investigate further the effect of ACTH on the incorporation of P^{32} into the RNA nucleotides of liver, an experiment was done using rats that had been adrenalectomized, hypophysectomized, or hypophysectomized and then adrenalectomized.

Table II shows the specific activities of each of the RNA nucleotides relative to that of the liver inorganic phosphate in the adrenalectomized rats. It can be seen that for each nucleotide the relative specific activity did not differ

TABLE I

RNA NUCLEOTIDES OF RAT LIVER

(Time after ACTH, 20 hr.; time after P^{32}, 16 hr.; mean ± S.E. mean)

Group	Specific activity relative to liver inorganic P × 10^2								
	Adenylic acid	*P*	Guanylic acid	*P*	Uridylic acid	*P*	Cytidylic acid	*P*	Average
Normal	29.3 ± 1.2 (23)[1]		25.0 ± 1.1 (24)		27.4 ± 1.1 (25)		24.9 ± 1.1 (23)		26.7
Hypophysectomized	18.7 ± 0.8 (7)	<0.001[2]	14.9 ± 0.7 (8)	<0.001[2]	17.0 ± 0.6 (9)	<0.001[2]	14.9 ± 0.8 (7)	<0.001[2]	16.4
Normal + ACTH	23.3 ± 1.1 (11)	<0.001[3]	19.6 ± 0.9 (11)	<0.001[3]	21.2 ± 1.0 (11)	<0.001[3]	19.5 ± 0.9 (11)	<0.001[3]	20.9
Hypophysectomized + ACTH	22.9 ± 0.8 (7)	<0.01[3]	20.3 ± 1.5 (8)	<0.01[3]	21.7 ± 1.4 (9)	<0.01[3]	19.6 ± 1.0 (7)	<0.01[3]	21.1

[1] *No. determinations.*
[2] *P value for hypophysectomized rats compared with normals.*
[3] *P value for rats receiving ACTH compared with similar animals receiving saline.*

TABLE II

RNA NUCLEOTIDES OF RAT LIVER

(Time after ACTH, 20 hr.; time after P^{32}, 16 hr.; mean ± S.E. mean)

Group	Specific activity relative to liver inorganic P × 10^2								
	Adenylic acid	*P*	Guanylic acid	*P*	Uridylic acid	*P*	Cytidylic acid	*P*	Average
Normal	35.3 ± 1.9 (6)[1]		32.0 ± 2.3 (5)		34.5 ± 3.3 (5)		30.7 ± 2.1 (6)		33.1
Adrenalectomized	38.3 ± 2.2 (8)	>0.3[2]	34.6 ± 2.4 (8)	>0.4[2]	41.1 ± 4.1 (8)	>0.2[2]	31.8 ± 2.1 (8)	>0.7[2]	38.6
Hypophysectomized	23.7 ± 1.7 (6)	<0.01[2]	19.7 ± 2.1 (6)	<0.01[2]	22.1 ± 1.9 (6)	<0.01[2]	21.0 ± 1.8 (6)	<0.01[2]	21.6
Hypophysectomized and adrenalectomized	24.5 ± 3.2 (7)	<0.02[2]	20.0 ± 2.8 (7)	<0.02[2]	22.9 ± 2.7 (7)	<0.05[2]	20.2 ± 2.7 (7)	<0.02[2]	21.9
Adrenalectomized + ACTH	36.9 ± 3.0 (9)	>0.7[3]	32.4 ± 2.9 (9)	>0.5[3]	33.8 ± 3.0 (9)	>0.1[3]	32.7 ± 3.1 (9)	>0.8[3]	34.0
Hypophysectomized and adrenalectomized + ACTH	17.8 ± 2.8 (8)	>0.1[3]	13.8 ± 2.3 (8)	>0.1[3]	16.2 ± 2.6 (8)	>0.05[3]	14.7 ± 2.7 (8)	>0.1[3]	15.6

[1] *No. determinations.*
[2] *P value for experimental rats compared with normals.*
[3] *P value for rats receiving ACTH compared with similar animals receiving saline.*

significantly from that of the normal animals, whereas for the hypophysectomized animals the incorporation was greatly reduced, thus confirming the result of the previous experiment (Table I). Adrenalectomy, when superimposed on the hypophysectomy, had no significant effect on the incorporation.

The administration of ACTH caused no significant change in the relative specific activities of the RNA nucleotides of the livers of the adrenalectomized animals. In the livers of the hypophysectomized–adrenalectomized rats that received ACTH, the mean specific activity of each of the nucleotides was decreased, but in no case was this decrease of statistical significance. It is noteworthy, however, that the decrease was uniform for all four nucleotides and amounted to 25–30%.

Discussion

Following hypophysectomy there is thus a decrease in the relative specific activity of each of the four RNA nucleotides of liver. This conclusive result confirms the observations made by Fraenkel-Conrat and Li (10), who used the method of Schneider (19) to estimate RNA, and by Riedel and DeLuca (18) in this laboratory, who used the procedure of Hammarsten (13) to isolate RNA.

The administration of ACTH affected the incorporation of P^{32} into the RNA nucleotides of the liver in both normal and hypophysectomized animals, but in opposite directions. From an average value of 26.7 in the normal group, the relative specific activity went *down* to 20.9 when ACTH was given, while in the hypophysectomized group from an average value of 16.4 it went *up* to 21.1 (Table I). As a result, there was no significant difference between the values for normal and hypophysectomized animals following administration of ACTH. In an attempt to control the effect of the operation, a further series of rats were subjected to a sham operation. In these sham hypophysectomized animals the average relative specific activity of the liver RNA nucleotides went *down* to a value of 20.3 after administration of ACTH, a value not significantly different from that found for the normal and the hypophysectomized animals. When these curious effects of hypophysectomy and of ACTH on the liver RNA phosphorus activities were found, further groups of adrenalectomized and adrenalectomized–hypophysectomized animals were studied in order to determine whether the effects were mediated by means of the adrenal gland.

In these experiments there was no significant change in the relative specific activity of each of the RNA nucleotides following adrenalectomy, either in normal or in hypophysectomized rats. When ACTH was given to the adrenalectomized animals there was not the usual decrease in the P^{32} incorporation and when the ACTH was given to adrenalectomized animals previously hypophysectomized, there was not the usual rise (Table II). These findings would suggest that the effect of ACTH on the incorporation of P^{32} into the liver

RNA nucleotides reported in Table I is mediated by means of the adrenal cortex in both normal and hypophysectomized animals. The fact that adrenalectomized animals do not show the decreased incorporation characteristic of hypophysectomy indicates that the situation must be rather complex and that the effect of hypophysectomy on the incorporation cannot be explained simply in terms of the deficiency of adrenal hormones that would result when the adrenal cortex is no longer stimulated by ACTH.

In interpreting the decrease in the relative specific activity of the liver RNA after hypophysectomy, a mechanism involving the adrenal glands would seem improbable, for adrenalectomy did not affect the relative specific activity of this constituent in either normal or hypophysectomized rats. However, injected ACTH did affect incorporation of P^{32} into the liver RNA. The observation that the effects of ACTH were in opposite directions in normal and hypophysectomized animals suggests that factors other than the adrenal cortical hormones are also involved. In addition, it is known that the energy intake of the diet (17, 22) and the thyroid status of the animal (20) influences the incorporation of P^{32} into liver RNA. Another factor to be considered is the concentration of RNA. No attempt was made to measure the RNA concentration in these studies, but the amount of RNA in the liver might change significantly during the course of the experiment, for hypophysectomy (1, 7, 12), adrenalectomy (1), growth hormone (7, 12), and ACTH (2) have all been shown to affect the liver RNA concentration.

A situation somewhat similar was encountered by Gemzell and Samuels (11) when they studied the specific activity of the inorganic phosphate of the liver 50 min. after the injection of P^{32}. Adrenalectomy did not affect the incorporation of P^{32} into the inorganic phosphate, but hypophysectomy caused a significant fall and normal levels were restored by a single injection of ACTH. If the situation is analogous to that reported here for the RNA nucleotides, the ACTH effect should be abolished by adrenalectomy. Unfortunately, this experiment was not done.

Gemzell and Samuels (11) suggested, as one possibility, that ACTH might influence the phosphorus metabolism of the liver by an effect other than that attributable to its action on the adrenal cortex. There is other evidence for such an extra-adrenal action for ACTH (14). However, we are inclined to reject this explanation in so far as the incorporation of P^{32} into liver RNA nucleotides is concerned. When normal or hypophysectomized rats were adrenalectomized, previously observed effects of ACTH on the incorporation of P^{32} into the liver RNA nucleotides were abolished. This finding would seem to indicate that the effects of ACTH on the formation of liver RNA nucleotides are secondary to the ACTH action on the adrenal cortex.

Acknowledgment

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A CHEMICAL PROCEDURE FOR DETERMINATION OF THE C^{14}-DISTRIBUTION IN LABELED GLUCOSE[1]

By B. Boothroyd[2], Stewart A. Brown, J. A. Thorn[3], and A. C. Neish

Abstract

A procedure based on the work of Jackson and Hudson was developed for the degradation of methyl-α-D-glucopyranoside formed from 2 millimoles of glucose. Oxidation of the glucoside with periodic acid gave C-3 as formic acid and a dialdehyde which was converted to the strontium salt of D′-methoxy-D-hydroxymethyl diglycolic acid. The purified salt was hydrolyzed to glyoxylic and glyceric acids. The glyoxylic acid was isolated as the 2,4-dinitrophenylhydrazone (C-1 + C-2); this was decarboxylated by heat to give carbon dioxide from C-2. The glyceric acid was oxidized by periodate to give C-4 as carbon dioxide, C-5 as formic acid, and C-6 as formaldehyde. This degradation permitted the determination of C^{14} in each position of the glucose molecule, the activity in C-1 being determined by difference. The method was applied to glucose-1-C^{14} and a sample of glucose labeled in all positions with satisfactory results.

Introduction

As radioactive tracer elements, especially C^{14}, are being used more frequently in the elucidation of metabolic pathways, more efficient methods for the degradation of labeled molecules to determine the distribution of the tracer element are necessary. In the case of a molecule such as glucose, which has six distinguishable carbon atoms, it is often difficult to measure the activity of each position separately. The chemical methods published so far for the degradation of glucose employ several different derivatives, and even then the activity in some atoms is obtained by difference (2, 5, 6). The same is true of the earlier biological methods (3, 4, 15) which by themselves did not give a complete analysis. A new method (9) based on the use of *Leuconostoc mesenteroides* shows promise of being very useful but is restricted to those hexoses which can be fermented by this organism.

The work of Jackson and Hudson on the periodate oxidation of methyl glycosides (11) indicates a method which should permit the degradation of any aldohexose, using a single sample of material, for determination of the activity in each of the six positions. Previous workers have used the periodate oxidation of methyl glycosides to determine the tracer in C-3 (5). We have developed the method, as outlined in Fig. 1, so that a complete analysis is possible, on a 1 millimole sample of methyl-α-D-glucopyranoside.

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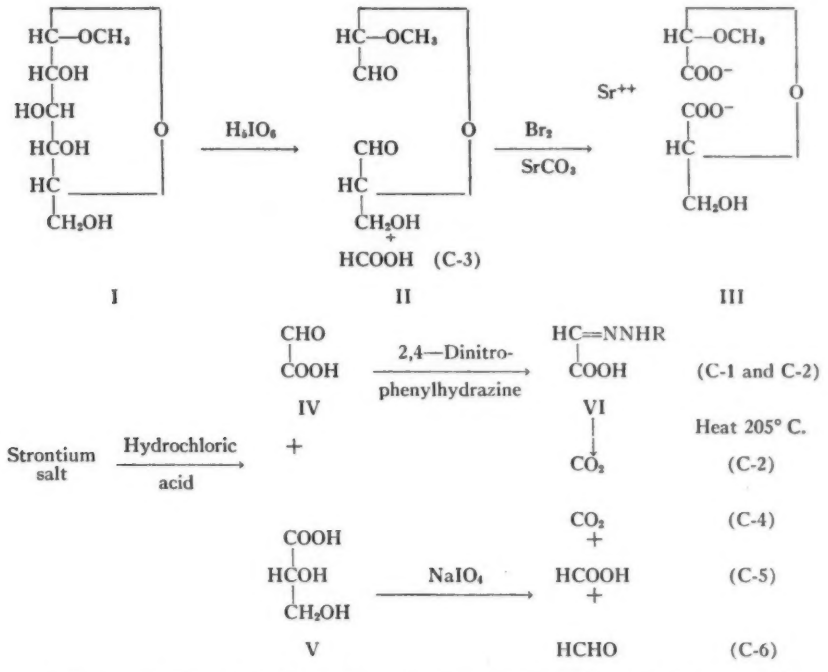

FIG. 1. Chemical degradation of methyl-α-D-glucopyranoside. R in (VI) is the 2,4-dinitrophenyl radical. C-1, C-2, etc., refers to the position of the carbon atom in the glucose molecule.

Experimental

Reagents

The Amberlite IR-4B resin was stirred with an excess of acetic acid (2 *N*) until effervescence had ceased and then washed three times with water by decantation, the fine particles being discarded. It was then transferred to a large sintered glass funnel, washed with water until free of acid, then washed with a large excess of ammonium hydroxide (1 *N*), and finally with water until the washings were neutral. This purified resin was stored under water; columns made from it were washed with water just before use. The Amberlite IR-120 resin was washed with hydrochloric acid, then with water until acid-free. Amberlite IRA-400 was washed with sodium hydroxide (*N*) and then with water until washings were neutral.

Glucose-1-C^{14} was prepared by the method of Isbell *et al.* (10). The sample of glucose labeled in all positions was obtained by hydrolysis of cellulose isolated from wheat plants that had been exposed to $C^{14}O_2$ (13).

The chromic acid reagent used for the complete combustion of organic samples was prepared according to directions given by Van Slyke and Folch (14).

Measurement of C^{14} in Carbonate Samples

Carbon dioxide, obtained from the oxidation of the various fractions as described below, was swept by a stream of carbon dioxide-free nitrogen into a bead tower containing sodium hydroxide (25 ml.; 0.2 *N*). This alkaline solution with the absorbed carbon dioxide was transferred directly to an apparatus similar to that described by Buchanan and Nakao (7), where it was acidified with sulphuric acid. This acidification released the carbon dioxide which was mixed with methane and counted in the proportional region. The counting assembly was standardized with the U.S. National Bureau of Standards C^{14} standard, a sodium carbonate solution rated at 1280 dps./ml. The precision of this method was quite good; duplicates agreed with ± 1%.

Preparation of Methyl-α-D-glucopyranoside

The methyl-α-D-glucoside was prepared by a modification of Fischer's (8) method. Anhydrous D-glucose (0.50 gm.) was mixed with a freshly prepared solution of hydrochloric acid in methanol (5 ml.; 3%). The mixture was heated at 100° C. for 1.5 hr. in a Leiboff urea tube. The solution was cooled to room temperature, mixed with three or four parts of water, and passed through a small column containing about 10 ml. of IRA-400 resin. The neutral effluent was evaporated in a current of air at room temperature to yield a sirup which crystallized on addition of ethanol giving 343 mgm. (64%) of the methyl-α-glucoside, m.p. 164–165° C. This was recrystallized from a mixture of equal parts of ethanol and methanol.

Periodic Acid Oxidation of Methyl-α-D-glucopyranoside

A solution of methyl-α-D-glucopyranoside (1 mM.) in water (3 ml.) was treated with 0.4 *M* periodic acid (5.5 ml.) at room temperature for three hours. The solution was then passed through a column of Amberlite IR-4B resin (1.9 × 6 cm.). The column was washed with distilled water until the total volume of eluate reached 110 ml. This eluate which contained the dialdehyde (II) (Fig. 1) was treated with bromine and strontium carbonate. The formic acid, representing C-3 of the glucose, was removed from the column with barium hydroxide. These operations, which were started without delay, are described below.

Determination of the C^{14} in C-3

After removal of the dialdehyde, the resin column was washed with a further 140 ml. of water, the washings were discarded, and the formic acid was eluted from the column with 0.25 *N* barium hydroxide (50 ml.) followed by water (50 ml.). The iodic and periodic acids remained on the column as the insoluble barium salts. The alkaline barium formate solution was acidified with 6 ml. of 85% phosphoric acid. The formic acid in this solution was then oxidized to carbon dioxide by refluxing for 1.5 hr. with mercuric oxide (3 gm.). The carbon dioxide was swept into a bead tower containing 0.2 *N*

sodium hydroxide (25 ml.) by a stream of carbon dioxide-free nitrogen. The carbonate solution was assayed for C^{14} as described above. This represents C-3 of the glucose; the recovery was about 80%.

Preparation of Strontium-D'-methoxy-D-hydroxymethyldiglycolate (III)

The neutral eluate containing the dialdehyde (II) was shown to be free from iodate and periodate by testing with potassium iodide and hydrochloric acid. It contained a trace of iodine which was formed by reaction of the resin with the iodic acid. Strontium carbonate (2 gm.) and bromine (0.4 ml.; 7.8 mM.) were added to the eluate, the mixture was stirred for 30 min., and the excess bromine then removed by a stream of air. Silver carbonate (2 gm.) was then added, the mixture stirred for 15 min. and then filtered to remove the silver carbonate, silver bromide, and strontium carbonate. The filtrate was buffered with strontium carbonate (1.0 gm.), treated for just one minute with a gentle stream of hydrogen sulphide to remove residual silver, immediately aerated for 30 min., and then filtered with Celite Analytical Filter-aid (product of Johns-Manville Co.). It is important not to prolong the hydrogen sulphide treatment. The slightly colored, neutral filtrate was concentrated to 15 ml. under reduced pressure at 40° C., treated with charcoal, and again filtered. The clear filtrate was concentrated on a steam bath in a stream of air to approximately 2 ml. and methanol (about 6 ml.) added until a cloudy solution resulted. Refrigeration caused crystallization of fine colorless needles. Seed crystals can be used to obtain more rapid crystallization and a cleaner product. This strontium salt was dissolved in about 5 ml. of water, the cloudy solution was filtered while hot, then treated with methanol (5 ml.) and allowed to crystallize in the refrigerator. The recrystallized product was washed successively with 50% methanol, methanol, and ether and then air-dried; 0.221 gm. (70%) of the dihydrate of III was obtained; $[\alpha]_D$ −56.4 ($c=1$, water).

Separation of Glyoxylic Acid (C-1, C-2) from Glyceric Acid (C-4, C-5, C-6)

The purified strontium salt (III) (0.221 gm.) was heated with 2 *N* hydrochloric acid (2 ml.) on a steam bath for one hour. The hydrolyzate was washed into 35 ml. of a solution of 2,4-dinitrophenylhydrazine (0.025 *M*) in hydrochloric acid (2 *N*). Precipitation of the hydrazone (VI) started at once. The precipitate was filtered out after one hour at room temperature, and washed with a little water. The filtrate, which contained the glyceric acid (V), was saved. The glyoxylic acid hydrazone (VI) was dissolved in sodium bicarbonate (10 ml.; 3.4%). This solution was filtered, and extracted with ethyl acetate (2 × 10 ml.) to remove neutral impurities. It was then treated with 2 *N* hydrochloric acid until acid to Congo red. The solution was filtered after one hour to give 0.124 gm. (70%) of purified hydrazone; m.p. 189–191° C. This was used for the determination of C^{14} in C-1 and C-2.

The combined filtrate and washings from the hydrazone (VI) were treated with Darco G-60 (1 gm.), filtered, the filtrate stirred for 20 min. with silver carbonate (12 gm.), again filtered and the filtrate passed through a column

(2.8 × 13.0 cm.) of Amberlite IR-120 resin to remove silver and strontium ions. The effluent was made slightly alkaline by addition of sodium hydroxide (*N*) and evaporated to approximately 20 ml. under reduced pressure at 40° C. This solution of sodium glycerate was used for the determination of C^{14} in C-4, C-5, and C-6, as described below.

Determination of C^{14} in C-4, C-5, and C-6

The slightly alkaline solution of sodium glycerate (see above) was made slightly acid by three drops of sulphuric acid (*N*), shaken for five minutes under the vacuum of a filter pump, and then buffered to pH 8 by addition of disodium hydrogen phosphate (5 ml.; 0.55 *M*). This solution was treated with sodium periodate (3.3 ml.; 0.4 *M*), the mixture being stirred for 30 min. at room temperature in a closed system. The mixture was then acidified with sulphuric acid (4 ml.; *N*) and the carbon dioxide (C-4; 90% recovery based upon the strontium salt (III)) swept out by a stream of carbon dioxide-free nitrogen into sodium hydroxide solution.

The reaction mixture was neutralized with excess calcium carbonate, filtered to remove the slightly soluble calcium iodate and periodate, and then reacidified with sulphuric acid (1.0 ml.; 10 *N*). This solution (50 ml.) was distilled at atmospheric pressure, the distillate together with formic acid and formaldehyde being collected in a receiver containing 15 ml. of a solution of dimedon in phosphate buffer. The buffer was prepared by adding sodium hydroxide (5.9 ml.; 1 *N*) to potassium dihydrogen phosphate (10 ml.; 1 *M*) and diluting the mixture to 100 ml. (pH 5.6–5.8); the dimedon (1.0 gm.) was then dissolved in this solution. The distillation was run until the volume was reduced to about 5 ml. and then water (10 ml.) was added and distillation continued until the volume was again reduced to 5 ml.; this was done twice. The distillate was then filtered to remove the formaldehyde–dimedon derivative. The precipitate (C-6; 86% recovery based on the strontium salt) was washed with water, recrystallized from ethanol–water, and then converted to carbon dioxide with Van Slyke's reagent, for measurement of C^{14}. The filtrate from the dimedon derivative, which contains the formic acid from C-5, was oxidized to carbon dioxide (76% recovery) by mercuric oxide in dilute phosphoric acid as described above for C-3.

Determination of C^{14} in C-1 and C-2

A portion of the hydrazone (0.080 gm.) was put in a small flask through which a stream of nitrogen could be passed. The flask was then immersed in a wax bath at 180° C., and the temperature raised quickly to 205° C. and held there for 20 min., while the carbon dioxide (C-2; 90% recovery based on the strontium salt) released by decarboxylation was swept by the nitrogen stream into sodium hydroxide as described above. The carbon dioxide was used for a direct determination of the specific activity of C-2. Another portion of the same sample of hydrazone (0.010 gm.) was completely oxidized by Van Slyke's reagent, the carbon dioxide being collected and its C^{14} content determined in order to find the amount in C-1 + C-2. The specific activity

of C-1 was then determined by difference. The material remaining in the flask following the decarboxylation was of a tarry nature, and several attempts to recover a pure compound representing C-1 were unsuccessful.

Results and Discussion

The procedure adopted (See Fig. 1) follows the work of Jackson and Hudson (11) up to and including the preparation of the strontium salt (III). However, in order that consistently good yields could be obtained on a 1 millimole scale, we found it necessary to modify their instructions, by reducing the time of periodate oxidation of the glucoside to three hours, and of bromine oxidation of the dialdehyde to 30 min., and also by using IR-4B resin for purification of the dialdehyde solution and recovery of the formic acid. A considerable amount of time was spent devising methods for the separation of glyoxylic and glyceric acids. Good separations were obtained by paper chromatography when phenol/acetic acid/water or *n*-propanol/ammonia /water were used, but it was not possible to obtain a good recovery of the glyoxylic acid from the paper. Partition chromatography on silicic acid with chloroform–butanol mixtures proved unsatisfactory because of the great tenacity with which the glyceric acid was retained by the column. The isolation of glyoxylic acid as the 2,4-dinitrophenylhydrazone was the most satisfactory of all the methods tried for the separation of glyceric and glyoxylic acids. It was found that the hydrazone (VI) could be easily decarboxylated by heat to give carbon dioxide, which is believed to be uncontaminated with carbon from the aldehyde carbon of the glyoxylic acid since glucose-1-C^{14} gave a derivative which evolved only inactive carbon dioxide on heating. The products obtained from the periodate oxidation of glyceric acid (V) are well known from previous work (12).

The procedure gave satisfactory results when applied to glucose-1-C^{14} (see Table I). The C-1 was found to have a specific activity close to the expected

TABLE I

DETERMINATION OF C^{14} DISTRIBUTION IN GLUCOSE

	D-Glucose-1-C^{14}		D-Glucose from wheat cellulose	
	μc./mM.	% of total	μc./mM.	% of total
Methyl-α-D-glucoside	2.01	100	0.2435	100
Strontium salt of diacid	1.98	98.4		
Glyoxylic acid 2,4-dinitrophenylhydrazone	1.96	97.5		
Carbon dioxide from:				
C_1	1.96	97.5	0.0414	17.0
C_2	Nil	Nil	0.0406	16.7
C_3	0.0139	0.69	0.0367	15.1
C_4	0.0030	0.15	0.0357	14.7
C_5	0.0040	0.20	0.0292	12.0
C_6	Nil	Nil	0.0510	20.9
			Total	96.4

value and it caused very little contamination of other fractions. In order to test this method thoroughly it would be necessary to have six different samples of glucose each labeled in a different carbon. We were not able to do this because of difficulty in obtaining such samples. However, the method was applied to a sample of glucose which was labeled in all six positions. It was found that the sum of the C^{14}-content of the six carbons determined separately was close to the total expected (Table I). This shows that there was no serious contamination of any of the fractions by inactive carbon picked up from the air or the reagents. This sample of glucose was isolated from the cellulose of wheat plants that had been exposed to $C^{14}O_2$ for a long period (13). It is of interest to note the non-uniformity of the labeling, which is particularly evident when comparing C-5 and C-6.

It might be of interest to mention another procedure that was tested. It was based on hydrogenation of the dialdehyde (II) to the corresponding triol, using conditions similar to those employed by Abdel-Akher *et al.* (1). The triol was hydrolyzed by acid to give a mixture of glycolic aldehyde and glycerol. These compounds were separated by partition chromatography on a Celite column and then degraded by periodate oxidation. This approach was abandoned because of heavy contamination of C-2 by C-1, probably due to enolization of the glycolic aldehyde during the hydrolysis.

As mentioned previously, the procedure described above is applicable to degradations on 2 millimole samples of D-glucose. This quantity permits the convenient isolation of enough barium carbonate from each of the six carbons for accurate C^{14} determinations. After a little experience, one operator can carry out a complete degradation to the point where all samples are ready for counting in about four days' working time, and several samples can be degraded concurrently.

Acknowledgment

The technical assistance of Mr. John Dyck, who made the radioactivity measurements, is gratefully acknowledged.

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SIGNIFICANT CHANGES IN THE LIPID AND WATER CONTENT OF THE THYMUS GLAND IN ALBINO RATS BEARING WALKER CARCINOMA 256[1]

By Eldon M. Boyd, Valmore Fontaine, and J. Gilbert Hill

Abstract

The investigation was designed to measure hydrolipotropic variations in the thymus gland of albino rats bearing Walker carcinoma 256. This was done upon 27 pairs of littermate albino rats, one of each pair inoculated and one not inoculated with Walker carcinoma 256. The life history of the tumor was evenly represented in the series. Tumor growth was found to be accompanied by a statistically significant increase in total body weight, due to water retention, and decrease in the weight of the thymus gland. The total amount of water, dry weight, total lipid, neutral fat, total fatty acids, free cholesterol, and phospholipid were significantly less in the thymus gland of tumor-bearing albino rats. The concentrations, per unit dry weight, of total lipid, neutral fat, and total fatty acids in the thymus gland were not significantly affected by tumor growth. The similar concentrations of water, total cholesterol, free cholesterol, and phospholipid were significantly increased in the thymus gland of tumor-bearing albino rats. These changes indicated a hydrolipotropic effect of the tumor upon the thymus gland. A pyramidal, up-and-down, change in the concentrations of phospholipid and the three cholesterol fractions in the thymus gland at T/RC coefficients of 30 to 60, together with a marked loss of weight by the gland, suggested the effect upon the thymus gland of factor(s) other than the hydrolipotropic influence.

Introduction

The investigation to be described below was an extension of studies upon lipid and water interrelationships in albino rats bearing Walker carcinoma 256 which have been in progress in the Department of Pharmacology at Queen's University since 1948. Walker carcinoma 256 is an experimental tumor originally discovered on February 12, 1928, by Dr. George Walker of Baltimore on the abdomen of a pregnant female rat and subsequently found to be an adenocarcinoma originating probably in a mammary gland (12). Initial studies by Walker and later by Earle (12–14) indicated that the tumor was transplantable in albino rats, gradually losing its adenocarcinomatous appearance and becoming, as confirmed in 1952 by Talalay, Takano, and Huggins (27), a highly anaplastic growth.

Previously reported (8, 9) changes suggested (7) "the production by the Walker tumor of a hydrolipotropic factor whose effect upon the tissues of the host is to make their water and lipid concentrations become more like those in the tumor component". The idea that cells of the host may become like those of the Walker tumor has been found expressed in one of Earle's original publications (13) in which he noted that a saline extract of the Walker tumor added to a tissue culture of rat mammary epithelium caused the nuclei of the fibroblasts to become like those of the tumor in microscopic appearance.

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Nakahara and his associates at the Cancer Institute in Tokyo have reported, in a series of papers published since 1948, the presence of a "toxohormone" in extracts of transplantable mouse sarcoma NF which can induce in normal mice many of the changes found in mice bearing this tumor (16). A similar concept has been indicated by Greenfield and Meister (17) and others as reviewed in 1954 by Greenstein from the U.S. National Cancer Institute (18) and by Begg at the first Canadian Cancer Research Conference (4).

Relevant Literature Upon the Thymus Gland

In 1951, Begg reported from Dalhousie University that there occurred a progressive decrease in the weight of the thymus gland in male Sprague-Dawley rats bearing Walker carcinoma 256 or the Jensen sarcoma (1). As noted by Begg, a similar involution of the thymus gland had been reported previously in tumor-bearing mice (20, 24). Begg reported later in 1951 that administration of testosterone and an extract of hog adrenals accentuated the loss of weight by the thymus gland in rats bearing Walker carcinoma 256 (2). Reporting from the University of Western Ontario in 1953, Stewart and Begg noted that force-feeding did not prevent loss of weight by the thymus gland of rats bearing Walker carcinoma 256 (26). Begg was inclined to relate decrease in weight of the thymus gland to increase in weight of the adrenal glands with a possible mediation through the pituitary gland (3, 4). An increase in the size of the adrenal glands of tumor-bearing albino rats has been noted but not measured during studies in the Department of Pharmacology at Queen's University. Fukuoka and Nakahara reported from Tokyo in 1952 that "the degree of thymus involution is plainly not correlated to the adrenal enlargement", in mice bearing sarcoma NF (15). Begg suggested inanition as another possible reason for loss of weight by the thymus gland (1). Apropos of this, Potter and his associates at the University of Wisconsin have found that fasting augments loss of weight by the thymus gland in female Sprague-Dawley albino rats bearing Flexner-Jobling carcinomata (22).

Mider and his associates reported in 1950 from the University of Rochester that lymphoid tissue (mixed lymph glands and thymus gland) from Wistar rats bearing Walker carcinoma 256 contained much less total nitrogen than was found in lymphoid tissue of pair-fed non-tumor-bearing animals (25). Busch has noted that the thymus gland of rats bearing 8- to 10-day Walker and other tumors converts acetate-1-C^{14}, injected intravenously, more readily to nonvolatile (an index of nonacetate) radioactivity than does the tumor (11). There is no significant difference between the ability of thymi from Akr mice bearing spontaneous lymphocytic leukemia to hydrolyze glycyl-glycylglycine and that of thymi from apparently healthy Akr mice, according to a report from McGill University by Birmingham and Grad (5). In none of these reports, except possibly the one from Rochester (25), was evidence given of significant changes in the chemical components of the thymus gland of tumor-bearing animals.

From the U.S. National Cancer Institute, Law reviewed in 1954 the genetic aspects of experimental cancer (21) and noted from his own work and that of others, particularly Furth, that thymectomy reduced the incidence and severity of experimental leukemia in mice. Autoplastic grafting of the thymus gland immediately after thymectomy restored the original susceptibility to experimental leukemia. Nakahara's group at the Japanese Cancer Institute reported in 1952 that injection of their toxohormone fraction of mouse sarcoma NF reduced the weight of the thymus gland but did not augment the weight of the adrenal glands in non-tumor-bearing mice (15).

This review of relevant literature indicates that the thymus gland has been proved to be affected to a considerable extent by, or to itself effect some of, the general tumor–host disturbances reviewed in 1954 by Greenstein (18). To the story as thus far unravelled, the present report adds information upon the lipid and water content of the thymus gland in albino rats bearing Walker carcinoma 256.

Littermate Analyses

The technique used was to analyze for lipids and water the thymus gland of littermate albino rats, one inoculated and one not inoculated in the groin two to five weeks earlier with Walker carcinoma 256, each pair being of the same sex. The weight of the tumor was estimated initially by observation as a percentage of host weight. Pairs of littermates were sacrificed in equal numbers at each 10% increment in tumor size as so estimated.

The albino rats were from a colony which has been bred in the animal quarters of the Department of Pharmacology at Queen's University since 1937 and were originally from a Wistar strain. No foreign rats have been in known contact with these albino rats which were under the supervision of an animal curator. They were fed Purina fox chow checkers and water ad libitum. An account of the technique of inoculation and the growth of Walker carcinoma 256 has been given in previous reports from this laboratory (8, 9). The present report is based upon analyses performed on 54 albino rats consisting of 27 littermate pairs sacrificed as noted above.

Each pair of littermates was sacrificed with chloroform. The Walker carcinoma 256 and observable secondary growths were dissected and weighed. Observable secondary growths were encountered in the postperitoneum, axilla, and occasionally in other subcutaneous regions, in albino rats bearing large primary tumors. The host was then weighed and the T/RC coefficient calculated after Boyd *et al.* (7) by dividing the weight of the tumor in grams by the weight of the host or residual carcass in grams and multiplying the resultant figure by one hundred. Most values for the T/RC coefficient fell between 5 and 100, one exceptionally large tumor yielding a T/RC coefficient of 152. A summary of the above data as obtained upon the 54 albino rats is given in Table I.

TABLE I

AGE AND SEX OF ALBINO RATS AND WEIGHT OF WALKER CARCINOMA 256

Observation	Mean	S. D.	No. of rats
Age of animals (days)	63.0	9.06	54
Sex of animals (% males)	63.0	—	54
Age of tumor (days)	25.5	7.59	27
Weight of tumor (gm.)	71.3	46.5	27
Weight of residual carcass (gm.)	158	42.5	27
T/RC coefficient	45.2	30.8	27

The thymus gland was dissected and weighed. An aliquot was dried to constant weight in a Fisher forced draft isotemp oven for the determination of water and dry weight. The remaining portion of the thymus gland was ground with sand to a homogenate which was extracted with alcohol–ether for lipid analyses after the oxidative micromethods of Bloor as modified by Boyd and associates (6, 10).

The number of littermate comparisons needed to prove significant any noted difference in mean values depended upon the extent of the mean difference and the percentage error of the analytical techniques. The limiting analytical error lay in the lipid techniques. The coefficient of variation in the lipid analytical techniques is of the order of two for analysis of not less than 2 mgm. of phospholipid, 1.5 mgm. of fatty acids, and 0.5 mgm. of free or total cholesterol. The optimal division of the alcohol–ether extracts of thymus gland found to yield approximately these proportions of lipids was 10% of the whole extract for total fatty acids, 40% for total cholesterol, and 50% for free cholesterol and phospholipid in the analytical scheme of Boyd (6). Many thymi, particularly from tumor-bearing albino rats, did not contain the above-noted minimal amounts of the several lipids. In these extracts, the analytical error increased. This required further repetition of littermate analyses until the significance of mean differences due to the presence of the Walker tumor were proved statistically significant or insignificant after the criteria of Bradford Hill (19).

Reduction of the Thymus

A statistical summary of all data upon body weight at the time of inoculation, final body weight, the weight of the thymus gland, and its per cent water is given in Table II. A significant finding was the loss of weight by the thymus gland in tumor-bearing albino rats, a loss which averaged approximately one half the weight of the organ. There was a significant increase in the per cent water content of the thymus gland in tumor-bearing animals, which indicated that the loss of dry weight was even more pronounced. Accompanying these changes in the weight of the thymus gland, there occurred a significant increase which averaged 27% in total body weight of tumor-bearing albino rats.

TABLE II

SIGNIFICANT CHANGES IN BODY WEIGHT AND IN WEIGHT OF THE THYMUS GLAND IN ALBINO RATS BEARING WALKER CARCINOMA 256

Observation	Values in non-tumor-bearing littermate controls (mean ± S.D.—27 rats)	Values in tumor-bearing rats (mean ± S.D.—27 rats)	Mean diff.	S.E. of mean diff.	*P* value of mean diff.	Significant % change in tumor-bearing rats
Body weight at time of tumor inoculation (gm.)	102 ± 27.7	109 ± 10.6	7	5.7	>0.05	
Final body weight (gm.)	181 ± 37.9	229 ± 63.4	48	14.2	<0.01	+27
Weight of thymus gland (mgm.)	353 ± 84.2	201 ± 140	152	31.0	<0.01	−43
Weight of thymus gland as per cent of body weight	0.190 ± 0.0571	0.0957 ± 0.0649	0.0943	0.0166	<0.01	−50
Water content of thymus gland—per cent of wet weight	79.1 ± 4.20	83.6 ± 5.36	4.50	1.32	<0.01	+ 5.7

In Fig. 1 has been charted a correlation of weight changes in the whole body and the thymus gland with increase in size of the Walker tumor. Having been plotted against the *T/RC* coefficient of the tumor-bearing host for which values were recorded, the weights of the thymus gland and total body weights were calculated as moving averages, three at a time, to illustrate correlative trends. There were no significant differences in the corresponding total

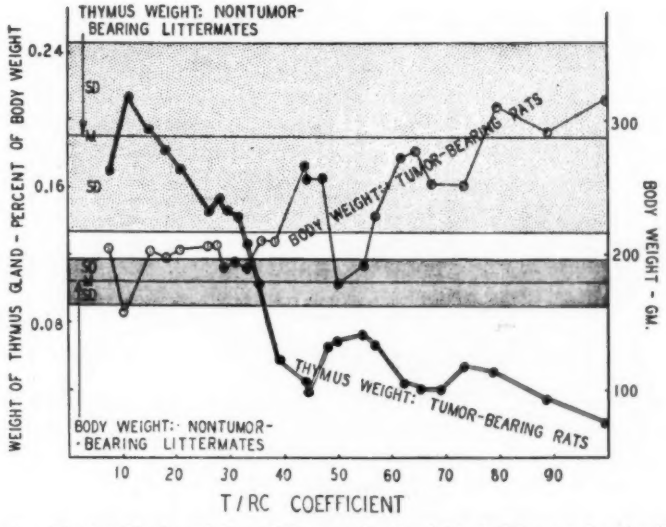

FIG. 1. The total body weight and thymus gland weight of albino rats bearing Walker carcinoma 256 in relation to tumor growth.

TABLE III

SIGNIFICANT CHANGES IN THE TOTAL AMOUNT OF LIPIDS, WATER, AND DRY WEIGHT IN THE THYMUS GLAND OF ALBINO RATS BEARING WALKER CARCINOMA 256

(The results are expressed as mgm. of component in the thymus gland per kgm. body weight)

Component analyzed	Values in non-tumor-bearing littermate controls (mean ± S.D.—(No. of rats))	Values in tumor-bearing rats (mean ± S.D.—(No. of rats))	Mean diff.	S.E. of mean diff.	*P* value of mean diff.	Significant % change in tumor-bearing rats
Total lipid	52.7 ±32.9 —(25)	30.0 ±31.3 —(21)	22.7	9.48	<0.05	−43
Neutral fat	34.0 ±30.4 —(25)	16.3 ±23.6 —(21)	17.7	7.97	<0.05	−52
Total fatty acids	44.7 ±29.8 —(27)	22.7 ±25.6 —(22)	22.0	7.92	<0.01	−49
Total cholesterol	3.41 ± 1.55—(27)	2.95 ± 1.58—(22)	0.46	0.45	>0.05	
Ester cholesterol	0.442 ± 1.51—(26)	0.588 ± 1.09—(21)	0.146	0.376	>0.05	
Free cholesterol	3.00 ± 1.14—(26)	2.25 ± 1.32—(22)	0.75	0.36	<0.05	−25
Phospholipid	18.5 ±11.9 —(26)	9.65 ± 8.48—(22)	8.85	2.96	<0.01	−48
Water	1480 ± 407 —(27)	788 ± 532 —(27)	692	129	<0.01	−47
Dry weight	408 ± 182 —(27)	771 ± 142 —(27)	237	44.5	<0.01	−58

body weights nor in the corresponding weights of the thymus gland of the non-tumor-bearing littermates, which have been entered in Fig. 1 as mean ± standard deviation. Mider, Tesluk, and Morton correlated total body weight with the age of Walker carcinoma 256 and demonstrated that body weight gain exceeds that of non-tumor-bearing control rats after some three weeks of tumor growth (23).

The data in Fig. 1 indicate that body weight of (apparently otherwise healthy) tumor-bearing albino rats significantly exceeds that of non-tumor-bearing littermates after the T/RC coefficient has reached 40 to 60. There is also the suggestion that rats bearing smaller tumors have a body weight relatively higher than that of their non-tumor-bearing littermate controls. The significance of these changes in body weight have been reviewed by Greenstein (18). A considerable proportion, if not all, of the gain in body weight by tumor-bearing albino rats can be proved due to increase in the water content of the tumor–host organism (7).

Reduction in weight by the thymus gland of tumor-bearing albino rats was probably present when the tumors were quite small and became statistically significant in the presently reported series at T/RC coefficients of 30 to 40. Note that the gradient of loss in weight by the thymus gland was more precipitous at T/RC coefficients up to about 50 and less precipitous as the tumor continued to grow beyond that point. The loss in weight by the thymus gland remains unexplained, except that it is correlated with tumor growth and perhaps in this case, as well as in mice bearing sarcoma NF, it may represent an action of tumor toxohormone(s) (15), or be secondary to adrenopituitary shifts (3, 4).

Weight reduction in the thymus gland of tumor-bearing rats was accompanied by a comparable loss of the total milligrams of water, dry weight, and most of the lipid components of the thymus gland as may be seen from the data assembled in Table III. There was no significant loss of ester cholesterol, there were insufficient data to prove any significant changes in the total amount of total cholesterol, while loss of the total amount of free cholesterol was only one half that of total thymus weight, water, dry weight, and the other lipid fractions. The losses of the cholesterol fractions were proved significantly less, at the $P < 0.05$ level, than the losses of the other components. These results indicated that there were varying alterations in the concentrations of these individual components in the thymus gland.

Water and Lipid Levels—Pyramidal Peaks

A statistical analysis of the concentrations of water and the seven lipid fractions, per unit dry weight of tissue, in the thymus glands of all tumor-bearing albino rats and in all non-tumor-bearing littermates, has been presented in Table IV. There were found to be statistically significant increases in the concentrations or levels of water, total cholesterol, free cholesterol, and phospholipid in the thymus gland of tumor-bearing albino rats. The

TABLE IV

SIGNIFICANT CHANGES IN THE WATER AND LIPID CONCENTRATIONS OF THE THYMUS GLAND IN ALBINO RATS BEARING WALKER CARCINOMA 256.

(The results are expressed as grams of component analyzed per 100 gms. dry weight of thymus gland)

Component analyzed	Values in non-tumor-bearing littermate controls (mean ± S.D.—(No. of rats))	Values in tumor-bearing rats (mean ± S.D.—(No. of rats))	Mean diff.	S.E. of mean diff.	*P* value of mean diff.	Significant % change in tumor-bearing rats
Water	399 ± 126 —(27)	528 ± 252 —(27)	129	54	<0.05	+32
Total lipid	15.4 ± 12.5 —(25)	17.5 ± 10.1 —(21)	2.07	3.32	>0.05	—
Neutral fat	9.88 ± 12.3 —(25)	6.72 ± 7.47—(21)	3.16	2.94	>0.05	—
Total fatty acids	12.5 ± 11.3 —(27)	11.8 ± 8.19—(22)	0.71	2.80	>0.05	—
Total cholesterol	0.963 ± 0.475—(27)	2.32 ± 2.33—(22)	1.36	0.503	0.01	+141
Ester cholesterol	0.142 ± 0.445—(26)	0.718 ± 1.48—(21)	0.576	0.319	>0.05	—
Free cholesterol	0.845 ± 0.442—(26)	1.50 ± 1.20—(22)	0.66	0.27	<0.05	+77
Phospholipid	4.78 ± 2.43—(26)	7.02 ± 4.80—(22)	2.24	1.13	0.05	+47

data were barely sufficient to prove an over-all change in phospholipid concentrations significant at the P 0.05 level. The data were insufficient to prove any over-all change in the level of ester cholesterol. As noted in Table IV, the variation in ester cholesterol levels was very high, the standard deviations exceeding their means. This was known to be due in part to two types of high analytical errors: *(a)* the level of ester cholesterol was the lowest of all lipids analyzed in the thymus gland and *(b)* values for ester cholesterol were claculated from determinations of total cholesterol and free cholesterol, thus admitting the possibility of a summation of analytical errors. There were no significant over-all changes found in the levels of total lipid, neutral fat, or total fatty acids in the thymus gland of tumor-bearing albino rats. The data assembled in Table IV indicated that a correlation of the levels of water, total cholesterol, free cholesterol, and phospholipid should be made with the T/RC coefficient in these tumor-bearing animals.

Such a correlation of water levels has been plotted in Fig. 2. The charted values for tumor-bearing albino rats and non-tumor-bearing littermates were calculated in the same manner as was done in preparing Fig. 1. The data indicated that water levels in the thymus gland of tumor-bearing albino rats progressively increased with increase in the T/RC coefficient.

A corresponding correlation of total cholesterol levels is presented in Fig. 3. In this charting, a new type of pyramidal, up-and-down, correlation was encountered. The pyramidal peak was due to results in seven tumor-bearing rats with T/RC coefficients from 31 to 59. The concentration of

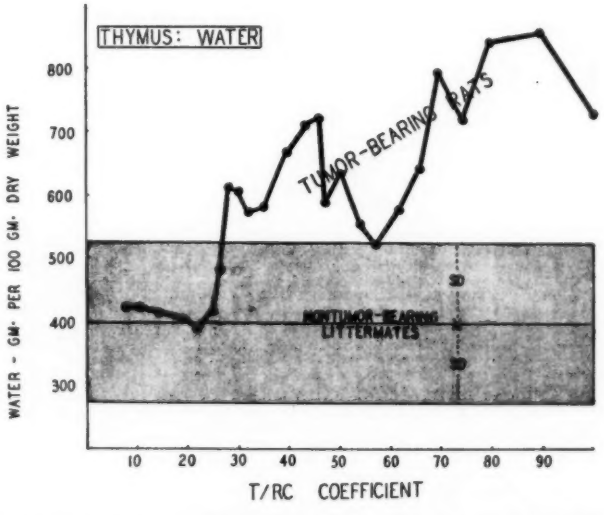

FIG. 2. Water levels in the thymus gland of albino rats bearing Walker carcinoma 256 correlated with increasing T/RC coefficients.

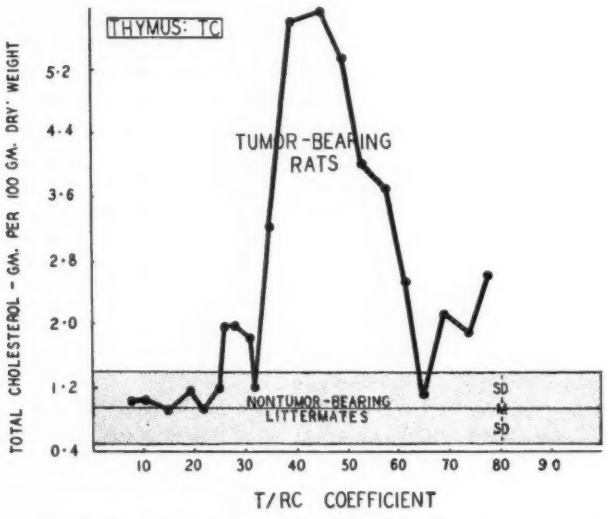

FIG. 3. Total cholesterol levels in the thymus gland of albino rats bearing Walker carcinoma 256 correlated with increasing *T/RC* coefficients.

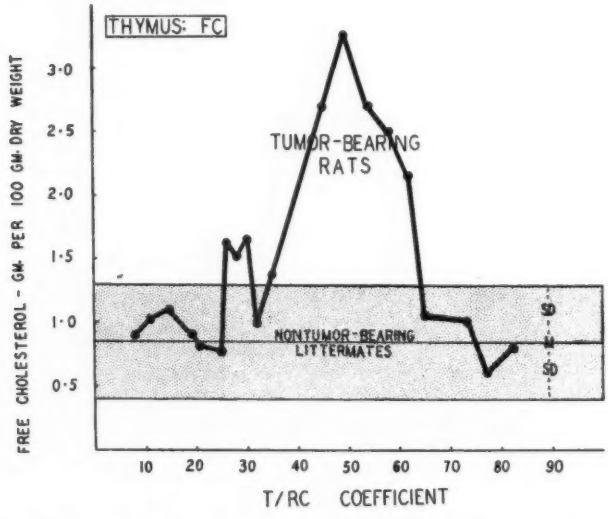

FIG. 4. Free cholesterol levels in the thymus gland of albino rats bearing Walker carcinoma 256 correlated with increasing *T/RC* coefficients.

total cholesterol in the thymus gland of these seven rats averaged 4.42 gm. per 100 gm. dry weight of thymus gland. The concentration of total cholesterol in the thymus gland of five tumor-bearing rats with *T/RC* coefficients of 60 and over averaged 1.58 gm. per 100 gm. dry weight of thymus gland. The mean difference has a *P* value of < 0.05. With the reservation that more tumor-bearing rats with appropriate *T/RC* coefficients must be examined to be certain of the conclusion, the results suggest that there is a significant pyramidal correlation between total cholesterol levels in the thymus gland of tumor-bearing albino rats and their *T/RC* coefficients. This in turn suggests a qualitative as well as quantitative alteration in cholesterol metabolism of the thymus gland with progressive growth of Walker carcinoma 256.

Changes in the level of free cholesterol in the thymus gland of tumor-bearing rats were then correlated in a similar manner with their *T/RC* coefficients and the results are presented in Fig. 4. A similar type of pyramidal, up-and-down, correlation, statistically significant even with the small number of rats involved in the pyramidal peak, was encountered.

The results observed in Figs. 3 and 4 suggested that a similar correlative plotting should be made for ester cholesterol levels, even though no statistically significant over-all mean change had been found (Table IV). Such was done and the data are given in Fig. 5. Again a pyramidal peak was noted, significant at the *P* 0.01 level in the eight rats involved.

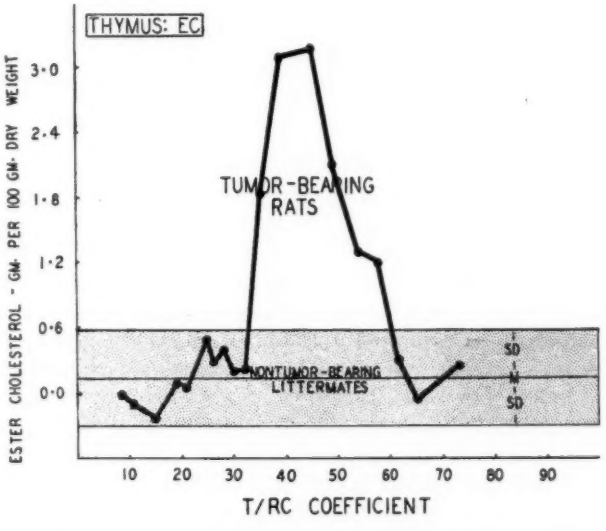

FIG. 5. Ester cholesterol levels in the thymus gland of albino rats bearing Walker carcinoma 256 correlated with increasing *T/RC* coefficients.

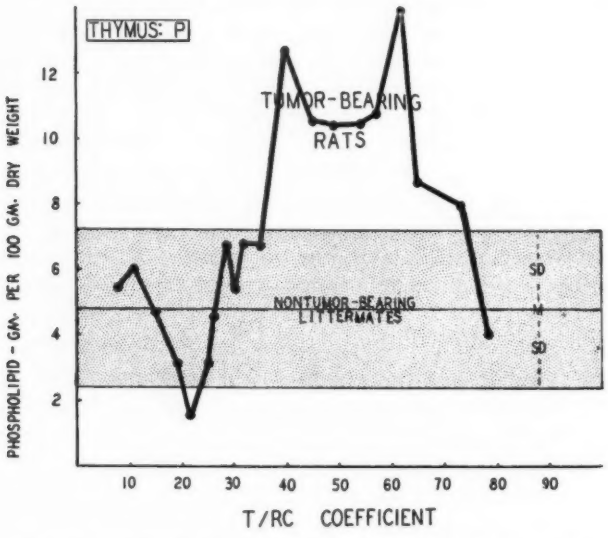

FIG. 6. Phospholipid levels in the thymus gland of albino rats bearing Walker carcinoma 256 correlated with increasing T/RC coefficients.

Finally, the same pyramidal peak was demonstrated in phospholipid levels and these data are presented in Fig. 6. A corresponding plotting of neutral fat values exhibited no significant correlation with the T/RC coefficient. Correlations of total lipid and total fatty acid levels with T/RC coefficients brought out lesser pyramidal peaks as could be predicted from the information given above.

Hydrolipotropic Correlations

The observed significant changes in the water and lipid content of the thymus gland in albino rats bearing Walker carcinoma 256 indicate that the thymus gland had cŏme under the hydrolipotropic influence of the tumor. Increases in the concentrations of water, phospholipid, and the cholesterol fractions in the thymus gland, toward the corresponding levels in the tumor, are indications of the hydrolipotropic influence of Walker carcinoma 256. These shifts are illustrated in Fig. 7. The decline in the mean level of neutral fat toward the low level in the tumor was not statistically significant.

The hydrolipotropic shift in the thymus gland toward the high levels of water, phospholipid, free cholesterol, and ester cholesterol, and the low levels of neutral fat and dry weight, in Walker carcinoma 256 is similar to that already shown to occur in studies reported from the Department of Pharmacology at Queen's University upon carcass, skeletal muscle, testicle, brain, heart, lung, liver, gut, and skin of the tumor-bearing albino rat (7, 8, 10).

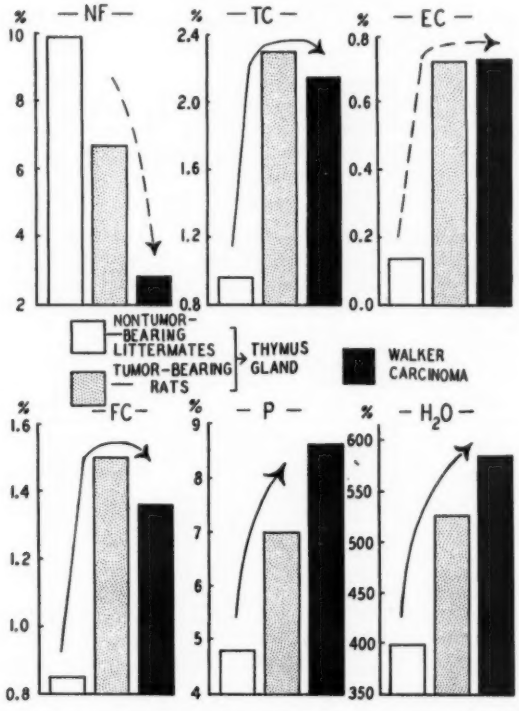

FIG. 7. The shift in concentration (gm. per 100 gm. dry weight) of lipids and water in the thymus gland of tumor-bearing albino rats toward levels in Walker carcinoma 256. Statistically significant shifts are indicated by arrows with solid lines, shifts of $P < 0.05$ by arrows with interrupted lines.

Legend: NF—neutral fat; TC—total cholesterol; EC—ester cholesterol; FC—free cholesterol; P—phospholipid; H_2O—water.

In this respect, the changes encountered in the thymus gland have been shown to be part of the general hydrolipotropic shift in tumor-bearing animals.

The pyramidal peaks, noted in the previous section, will require further investigation of their significance. If their presence is confirmed, evidence of an influence other than the hydrolipotropic shift will be proved present in the thymus gland of tumor-bearing albino rats, the second influence(s) being further indicated in the marked loss in weight of the thymus gland.

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THE ROLE OF ALKALINE PHOSPHATASE IN INTESTINAL ABSORPTION

III. THE EFFECTS OF VARIOUS FATTY ACIDS ON LEVELS OF THE ENZYME IN INTESTINAL MUCOSA[1]

By Nester Dickie, Margaret I. Robinson, and Jules Tuba

Abstract

Fasted rats were used in a study of the effect of fatty acids on intestinal and serum phosphatase. Each acid was mixed with a palatable carrier, and then offered to the animals. Enzyme estimations were made with intestinal homogenates, and occasionally with serum. The first carrier, casein, was found to affect intestinal phosphatase activity, and it was replaced by a non-active carrier, wheat gluten. The results with the two carriers showed essentially the same trend. The elevation of intestinal alkaline phosphatase by dietary fatty acids varied inversely with the chain length for: butyric acid, lauric acid, palmitic acid, and stearic acid. The possibility is considered that decreasing solubility may be associated with the response obtained with these fatty acids. Oleic acid was a more powerful stimulant for the synthesis of intestinal alkaline phosphatase than the saturated fatty acids. Choline, fed simultaneously with oleic acid, completely obliterated the highly significant effect of the acid on both the intestinal and the serum enzyme, These findings indicate that further consideration should be given to the role of phospholipids in absorption of fatty acids. Enzyme response in the serum, studied with a more limited number of fatty acids, lagged a few hours behind the response of the intestinal enzyme.

Introduction

There is no current theory for the absorption of fats from the intestine which is completely acceptable to everyone. Evidence has been brought forward by a number of workers which seemed to indicate the formation of phospholipids during the absorption of the products of fat digestion (2, 5, 16). On the other hand, Zilversmit, Chaikoff, and Entenman (24) found no increase in the amount or the turnover of phospholipids in the mucosa or villi of the small intestine. It has been demonstrated, however, that serum and intestinal phosphatase levels are significantly elevated following the ingestion of fats (4, 6, 8, 12, 13, 17, 21, 22, 23). It is also known that the elevation in values of the enzyme in serum represents the translocation of alkaline phosphatase from the intestinal mucosa, where the phosphatase has been formed in response to the feeding of fat (13, 21). Since alkaline phosphatase is known to function in a number of tissues as a dephosphorylating enzyme, and because it has been shown to participate in the absorption of certain monosaccharides from the intestine (18), it seems probable that at least some fat is absorbed by mechanisms which involve phosphorylation-dephosphorylation. Support for this view is found in the work of Kornberg and Pricer (10, 11) which shows that α-glycerol phosphate is esterified with fatty acyl coenzyme A derivatives.

Previously we have considered the effect, on intestinal and serum alkaline phosphatase, of dietary fats, such as Crisco, (a hydrogenated vegetable fat) (13) and olive oil (21). Taylor, Madsen, and Tuba (17) studied the effects of oleic acid and stearic acid on the serum enzyme. Because changes in the

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serum enzyme represent changes which originally occurred in the intestinal mucosa, the work with fatty acids has now been extended to include the effect on the intestinal enzyme.

Experimental

Mature, male rats, which weighed 250-350 gm. each were selected. They were housed in separate cages, and in order to standardize conditions the animals were given water and the standard laboratory diet of Purina fox checkers ad libitum for a week before the experiment. They were then deprived of food for four days. On the fourth day, the experimental diet was placed before each animal at 4.30 p.m., in 5-gm. portions. The animals were killed by decapitation 6-24 hr. after food was offered to them; the variation in time will be discussed below. Ten centimeters of the intestine from the pylorus was removed from each animal and homogenized with a Potter glass homogenizer as previously described (18). In some of the experiments blood was collected at the time of decapitation for estimation of levels of serum alkaline phosphatase. Levels of the enzyme in intestinal homogenate and in serum were established by the micromethod of Shinowara, Jones, and Reinhart (15), as modified by Gould and Schwachman (9). The unit of phosphatase activity is defined as equivalent to 1 mgm. of phosphate ion liberated during one hour of incubation at 37° C. with a substrate containing sodium β-glycerophosphate, hydrolysis not exceeding 10% of the substrate, and at pH 9.3 ± 0.15 (13).

In experiments on the effect of dietary carbohydrate on intestinal phosphatase, we found that one of the difficulties was the matter of inducing the animals to eat the sugars (18). This was solved by the use of non-nutritive, finely powdered cellulose as a carrier for the sugars. Although cellulose alone or mixed with a digestible carbohydrate was eaten readily by the fasted animals, a mixture of cellulose and a fatty acid was usually sufficiently repellent to fasted rats to result in very unsatisfactory consumption of the diets. The choice of a suitable carrier for the fatty acids is discussed below.

Diets Containing Casein, Fatty Acids, and Choline

Under experimental conditions quite different from these investigations with fasted rats, it had been observed that dietary casein had no effect on levels of serum alkaline phosphatase (19). It was assumed, since serum values of the enzyme are affected by activity in the intestinal mucosa, that casein could be used as a carrier for fatty acids, without influencing enzyme levels in the serum or intestine. Hence alterations of enzyme activity could be ascribed to the fatty acid itself. It was noted, moreover, that the rats found a mixture of casein and fatty acid more palatable than when cellulose was used as a carrier. Consequently a number of observations were made with diets containing casein and fatty acids and, in some instances, choline. Subsequently, in the course of a study of the effect of various proteins on intestinal phosphatase levels, it was observed that casein was able to modify

intestinal phosphatase activity. Hence some of our experiments were later repeated with the use of gluten, one of the proteins that were found to have no influence on the values of the intestinal enzyme.

In the first experiment, five groups of four rats each were used to test the effects of the diets indicated in Table I. Fatty acids were intimately mixed with casein in a mortar and each 5 gm. portion of the diet contained 0.00035 gm-moles of fatty acid (7). Choline chloride, when it was added, was mixed in the same way and at two concentrations: 5 mgm. per 30 gm. diet (7), and 10 mgm. per 30 gm. diet. The animals were sacrificed 24 hr. after each one had been given a 5 gm. portion of the appropriate diet. The levels of intestinal phosphatase were determined in the usual way and are given in Table I. The experiment was repeated with seven groups of four animals each, and two levels of choline were used with oleic acid and stearic acid. With these animals, the levels of serum alkaline phosphatase were determined, and the results are incorporated into Table I.

TABLE I

THE EFFECT OF DIETARY CASEIN, FATTY ACIDS, AND CHOLINE UPON THE INTESTINAL AND SERUM ALKALINE PHOSPHATASE OF FASTED RATS

Diet	Intestinal alkaline phosphatase (units/100 gm. wet weight)	Serum alkaline phosphatase (units/100 ml.)
A. Casein	9980±525*	35.1±1.8
B. Stearic acid	12,700±1660	45.3±4.4
C. Stearic acid + choline (5 mgm.)		44.5±6.2
D. Stearic acid + choline (10 mgm.)	12,200±1300	45.3±1.3
E. Oleic acid	18,200±2000	60.1±5.9
F. Oleic acid + choline (5 mgm.)		40.4±5.0
G. Oleic acid + choline (10 mgm.)	14,600±1350	38.7±2.3

* *Standard error of the mean.*

It should be recalled that the enzyme values for the intestine and for the serum were done on different groups of rats. However, Diets A, B, and E were fed to three groups of animals, the enzyme was determined in serum and intestine of the same animal, and results essentially the same as above were obtained.

From the data in Table I it is evident that oleic acid produced a highly significant elevation of alkaline phosphatase in the intestine and in the serum ($P<0.01$). By contrast, stearic acid appeared to have a slight but statistically insignificant effect upon both the intestinal and serum enzyme, when the phosphatase values were compared with those of the animals fed casein alone. Supplements of choline in the diet produced a diminution in the enzyme response to oleic acid in both the serum and the intestine; and the two levels of choline used had much the same effect. In the serum the effect of choline was significant ($P<0.01$), and in the intestine it was at the level of significance ($P<0.05$). Choline supplements added to stearic acid in the diet were without effect. The effect of choline on alkaline phosphatase of the serum of rats has some support in the findings of Tuba and Madsen

(20). They noted that supplements of choline added to a basal diet of Purina fox checkers produced a highly significant ($P<0.01$) lowering of 20% in the enzyme of adult male rats in spite of an increase in body weight during the experimental period of 14 days.

Preliminary work had led us to suspect that there might be a difference between the time required to obtain the maximum response to fatty acid and to choline in the serum and in the intestine. The next experiment was designed to establish clearly the optimum time for killing the animals after they had been fed. The number of diets was limited to three: a casein control, oleic acid plus casein, and oleic acid plus casein supplemented with choline.

Three groups of animals were selected and treated exactly as in the experiments above. The concentration of the oleic acid was the same in each diet. Choline was added as a supplement in one diet at a level of 10 mgm. per 30 gm. of diet. A group of three fasting rats was killed for control values at 4.30 p.m. at the beginning of the feeding period. Four animals from each of the three groups receiving the diets above were killed at 6, 12, 18, and 24 hr. Serum and intestinal enzymes were estimated in the usual way, and the results are presented in Table II.

TABLE II

THE EFFECT OF FEEDING CASEIN, OLEIC ACID, AND CHOLINE ON THE INTESTINAL AND SERUM ALKALINE PHOSPHATASE OF FASTED RATS AT 6, 12, 18, AND 24 HR. AFTER FOOD WAS OFFERED

(Values are means of four rats except fasting group which is mean of three)

Time, hr.	Diet	Intestinal alkaline phosphatase, (units/100 gm.)	Serum alkaline phosphatase, (units/100 ml.)
0	Fasting	6540 ± 756*	37.7 ± 1.1
6	Casein	19,500 ± 1730	63.6 ± 10.1
	Casein + oleic acid	24,000 ± 2000	76.0 ± 4.8
	Casein + oleic acid + choline	18,000 ± 3390	58.9 ± 2.4
12	Casein	13,100 ± 1470	67.5 ± 13.5
	Casein + oleic acid	15,600 ± 1330	90.2 ± 1.1
	Casein + oleic acid + choline	13,200 ± 583	83.8 ± 3.6
18	Casein	9540 ± 740	62.3 ± 6.7
	Casein + oleic acid	11,500 ± 2180	59.7 ± 1.7
	Casein + oleic acid + choline	8400 ± 1260	56.1 ± 3.4
24	Casein	11,000 ± 1930	39.6 ± 1.1
	Casein + oleic acid	9940 ± 2170	61.0 ± 2.4
	Casein + oleic acid + choline	8340 ± 1100	57.6 ± 8.6

* *Standard error of the mean.*

It appears from Table II that the response of intestinal alkaline phosphatase to all three diets was greatest six hours after the animals were offered food. On the other hand, the maximum effect of the diets on serum alkaline phosphatase was delayed for another six hours. It is apparent also that casein,

considered to be without effect by previous workers (19), produced a highly significant elevation of the intestinal and serum phosphatases from the fasting levels. This finding necessitated the use of another and more suitable carrier for the fatty acids. Wheat gluten was finally chosen because it was readily eaten by the animals and it did not elevate the levels of the alkaline phosphatase above fasting values (cf. next paper of series). The question of the response of intestinal alkaline phosphatase to the ingestion of fatty acids was then re-examined.

Diets Containing Gluten, Fatty Acids, and Choline

Ten groups of mature, male animals were chosen and treated as in the previous experiments. A number of fatty acids were incorporated singly into wheat gluten in a concentration of 0.00035 gm-mole per 5 gm. of diet. To one diet which contained oleic acid, choline was added at the rate of 10 mgm. per 30 gm. It will be noted in Table III that there were used three groups of control animals, which were fed 5 gm. of gluten alone. Three groups were used because, with the large groups we used, it was impossible to complete the experiment in one day, and gluten controls were used on each of the three days required. One diet had choline added to the gluten alone, to test the effect of choline in the absence of fatty acid. At 4.30 p.m. on the fourth day of fast, a 5 gm. portion of each diet was offered to each rat in the group. After six hours, the animals were killed, and the intestine was removed from each and homogenized in the usual way. It should be noted that the animals were fed at 4.30 p.m. because they eat better then than they do earlier in the day. By the time the animals were killed and the usual portion of intestine was removed from each, it was often too late to homogenize all samples, and in such an event the intestinal tissues were frozen overnight, with no loss of enzyme activity.

TABLE III

EFFECT OF FEEDING VARIOUS FATTY ACIDS AND CHOLINE, EACH WITH WHEAT GLUTEN, UPON INTESTINAL ALKALINE PHOSPHATASE LEVELS OF FASTED RATS

No of rats	Diet	Alkaline phosphatase (units/100 gm.)	t	P
7	Butyric acid	7060 ± 532*	3.3**	<0.01
7	Lauric acid	6730 ± 506	2.92	<0.02
4	Gluten control	4860 ± 392		
7	Palmitic acid	6780 ± 415	2.83	<0.02
7	Stearic acid	5830 ± 792		
6	Gluten control	5000 ± 469		
12	Oleic acid	8380 ± 685	4.83	<0.01
8	Oleic acid + choline	3510 ± 436		
8	Choline	4610 ± 708		
8	Gluten control	5940 ± 566		

* *Standard error of the mean.*
** *Obtained by comparison with its gluten control group.*

The data in Table III show that there appears to be an inverse relationship between chain length of fatty acid and its effect on intestinal alkaline phosphatase, except in the case of oleic acid, where the existence of the double bond resulted in the greatest response by the enzyme. Choline in the absence of oleic acid does not significantly alter the phosphatase values from those obtained for gluten alone.

A number of points in the above three tables are worthy of further comment. Once more the early work of Weil and Russel (23) is borne out in that the effect of dietary fatty acids on serum alkaline phosphatase is very markedly evident. In addition we observed a profound stimulus to the formation of the intestinal enzyme. The decreased effect of fatty acids as their chains lengthen is of interest. One of the reasons for this may be slower absorption of the longer chained acids. It is evident in the data above that stearic acid produces a greater effect on the intestinal enzyme in 24 hr. than in six hours, although these differences are not statistically significant. Bloom, Chaikoff, and Dauben (3) found that stearic acid fed to rats was absorbed slowly, 35-54% in 19-23 hr. However, the rapid and pronounced effect of oleic acid in six hours on the intestinal enzyme may be associated with other factors such as its liquid state or the pathway and mechanism of absorption from the gut. The ability of dietary choline supplements to decrease significantly the effect of oleic acid on intestinal alkaline phosphatase has been observed in a number of experiments over some years in this laboratory and it merits further investigation. Artom and Cornatzer (1) believe that choline is a limiting factor in phospholipid formation during the absorption of fat. Platt and Porter suggest that choline exerts a mass action effect on phospholipid turnover (14). In the light of these observations it may eventually be possible to correlate this effect of choline with some aspect of phospholipid turnover.

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THE ROLE OF ALKALINE PHOSPHATASE IN INTESTINAL ABSORPTION

IV. THE EFFECTS OF VARIOUS PROTEINS ON LEVELS OF THE ENZYME IN INTESTINAL MUCOSA[1]

By Jules Tuba and Nester Dickie

Abstract

Fasted adult male rats were used to study the effect of dietary proteins on intestinal alkaline phosphatase. Groups of animals were offered one of several proteins; lactalbumin, egg albumin, zein, gelatin, wheat gluten, casein, and vitellin. Control animals had cellulose fed to them. The rats were sacrificed six hours after they were given the different diets. Alkaline phosphatase determinations with intestinal homogenates indicated that the two phosphoproteins, casein and vitellin, elevated levels of the enzyme significantly above fasting levels. Possible interpretations of these findings are discussed.

Introduction

A highly significant effect of dietary casein upon the intestinal alkaline phosphatase of fasted rats was noted in the preceding paper (3). This was unexpected because previous experiments in this laboratory had shown that casein had no significant effect on serum alkaline phosphatase levels (13). Weanling rats had been fed diets which contained 0, 5, 10, 30, or 91% of the protein, and while a highly significant correlation existed between fat consumption and enzyme activity, this was not true for consumption of casein. In addition, adult rats, after a three week protein depletion period, were subjected to a two week period of repletion by several proteins, including casein. Once more variations in enzyme activity could be attributed directly to the fat component of the diets, and not to any of the proteins. However, Lawrie and Yudkin (6) found that a 70% casein diet produced an elevation of the intestinal alkaline phosphatase activity of the same order as the effect of a 70% lard diet.

It is probable that the effect of casein on the intestinal enzyme is associated with some of the structural components of its molecule. There is the possibility that one or more of the amino acids liberated during digestion may be absorbed in the gastrointestinal tract by an active process which involves the action of alkaline phosphatase. This possibility is being explored at the present time. Then again the intestinal enzyme could be affected by the presence in the casein molecule of the prosthetic group, orthophosphoric acid which is esterified with the β-hydroxy group of serine (7).

The experiments reported in this paper were designed to test the effect of different dietary proteins upon the alkaline phosphatase of fasted rats. Another phosphoprotein, vitellin, was included for comparison with the effect of casein.

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Experimental and Results

An initial experiment was carried out with two proteins to find the optimum time for killing the rats after they were fed. For this purpose we used casein, which had been used as a carrier for fatty acids in previous work (3), and which was known to have an effect on intestinal phosphatase activity. In addition lactalbumin was used for comparative purposes.

Three groups of mature male animals were fasted for four days during which time they received water ad libitum. On the fourth day of the fast, at 4.30 p.m., one group of rats was offered 5 gm. of casein each and another group received 5 gm. of lactalbumin each. A control group was given 5 gm. per rat of non-nutritive, finely powdered cellulose, which was known to have no effect on fasting intestinal alkaline phosphatase levels (12). Two animals from the control group were killed at once by decapitation. Two animals from each of the three groups were killed 3, 6, and 15 hr. after food had been placed before them. Alkaline phosphatase values were determined in intestinal homogenates as previously described (12) by the micromethod of Shinowara, Jones, and Reinhart (10), as modified by Gould and Schwachman (5). The unit of phosphatase activity is defined as equivalent to 1 mgm. of phosphate ion liberated during one hour of incubation at 37° C. with a substrate containing sodium β-glycerophosphate, hydrolysis not exceeding 10% of the substrate and at pH 9.3 ± 0.15.

The results of the above experiment are given in Table I. Once more a very pronounced elevation of intestinal alkaline phosphatase was noted six hours after the rats had received the casein diet (cf. preceding paper (3)). Lactalbumin did not elevate enzyme levels above fasting values which are characteristic for the animals which had been fed cellulose.

In the next experiment the effect of a number of dietary proteins upon the intestinal alkaline phosphatase activity of fasted rats was investigated. The animals were fasted for four days before the tests. Again, at 4.30 p.m., 5 gm. portions of the various proteins were placed before each animal, except the controls which were given 5 gm. of cellulose each. It was necessary to break the experiment up into several days because of the large number of animals

TABLE I

THE EFFECT OF CASEIN AND LACTALBUMIN ON THE INTESTINAL ALKALINE PHOSPHATASE ACTIVITY OF FASTED RATS, 3, 6, AND 15 HR. AFTER FEEDING

(Each value is the mean for two rats)

Diet	Intestinal alkaline phosphatase (units/100 gm.)			
	0 hr.	3 hr.	6 hr.	15 hr.
Cellulose	3080	3260	2880	3000
Casein		2410	7200	7280
Lactalbumin		2810	3980	2900

involved. Controls were provided every day, because the slight daily variations observed in these, or even in fasted animals, could possibly obscure changes which were not pronounced. On the basis of the first preliminary experiment reported in this paper, and in view of our experiences with the absorption of carbohydrates (12) and of fatty acids (3), six hours was chosen as the most suitable time for killing the rats after they had been offered the different proteins or cellulose. Almost invariably the animals had consumed all the diet in this time, but if they had not they were rejected from the experiment. Intestinal alkaline phosphatase values for these animals are given in Table II.

TABLE II

THE EFFECT OF VARIOUS DIETARY PROTEINS ON INTESTINAL ALKALINE PHOSPHATASE OF FASTED RATS

Diet	Number of rats	Intestinal alkaline phosphatase (units/100 gm.)
Casein	7	6180 ± 614*
Control	5	3470 ± 350
Lactalbumin	18	4580 ± 303
Control	12	4270 ± 357
Zein	10	4540 ± 347
Egg albumin	9	3670 ± 401
Control	11	4050 ± 355
Gelatin	4	5540 ± 541
Control	4	4740 ± 439
Wheat gluten	6	3730 ± 541
Control	8	3420 ± 438
Vitellin	5	6120 ± 518
Control	9	3900 ± 223

* *Standard error of the mean.*

There was no statistically significant effect on activity of the serum enzyme produced by lactalbumin, egg albumin, zein, gelatin, or wheat gluten. The two phosphoproteins, casein and vitellin, produced highly significant elevations of enzyme activity. The enzyme level for the casein group compared with its control gave a value of $t = 3.93$ ($P < 0.01$). The experiment with vitellin was repeated once more and essentially the same effect was observed.

Discussion

The two phosphoproteins, casein and vitellin, were the only two, of the seven proteins tested, which affected intestinal alkaline phosphatase activity of starved rats. Elevation of the low fasting levels would point to the possibility that the prosthetic group of these two proteins might be involved in

the response which they produced. Perlmann (9) has shown that α-casein is readily dephosphorylated by prostatic phosphatases at pH 5.6–6.6. Phosphoprotein phosphatases have been found in a number of other tissues including ox-spleen (11) and stomach mucosa (8) and in various tissues of the rat including the intestine (4). The purified enzyme from stomach mucosa acted on phosphopeptone, casein, and α-casein but it was inactive with β-glycerophosphate. Therefore it seems unlikely that the intact casein molecule was the stimulus for increased intestinal alkaline phosphatase activity of the rat.

Active transport of some amino acids against a concentration gradient has been demonstrated by Agar, Hird, and Sidhu (1,2), who used isolated loops of rat intestine. Exploratory experiments in this laboratory have indicated that single dietary amino acids may affect levels of intestinal alkaline phosphatase. This suggests that the active absorption of some amino acids may involve the action of phosphatase. However, if it has no other action during casein digestion, intestinal phosphatase in all probability dephosphorylates serine phosphate.

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THE STABILIZATION OF THE FLUORINE CONCENTRATION OF THE TOTAL ASH OF RATS[1]

By Sanford H. Jackson

With the technical assistance of Dorothy Train

Abstract

Rations containing 0.5 and 1.0 mgm. % of added fluorine were fed to growing rats for six months. Representative groups of animals were killed at monthly intervals and analyzed for total fluorine and ash. The fluorine reached a constant concentration in the ash after the rats had been kept two to three months on the 0.5 mgm. % ration and five months on the 1.0 mgm. % ration. Young rats on a low calcium - high fluorine ration stored more fluorine than rats on a similar ration with added calcium. Two groups of young rats consumed the same total amount of fluorine over an eight week period. The first group received the fluorine mixed with the basal ration which was consumed ad libitum. The second group received the basal ration alone and in addition, on one day each week, the amount of fluorine ingested by the first group during the previous week. The fluorine retention was the same in both groups so that irregularity of ingestion had no effect.

Previous experiments (1) have indicated that when rats are fed a ration, containing a constant proportion of fluorine, the element accumulates until after about six months, a level of total fluorine in the rat carcass is attained that is not further increased even though the ration is still consumed for a year longer. In these experiments, the first determination of total fluorine stores was made at six months, at which time they were found to be at or near maximum. It therefore became of interest to study the rate of accumulation of fluorine during this initial six month period. We suggested, in our previous report (1), that the accumulating stores of fluorine in the body caused the rate of excretion of fluorine to increase until no further fluorine was stored and the body content became constant. This suggested that if fluorine were to be consumed in a few relatively large doses it could lead to less accumulation in the body than if the same amount were consumed in a larger number of small doses over the same period of time, since, if excretion was sufficiently rapid, the fluorine from each dose in the former case might be almost all excreted before the next dose was ingested. This point is of some interest in considering the human consumption of fluorine in food, as opposed to that in domestic water supplies. Most of the items in the human diet contain very small amounts of fluorine but there are some, such as canned salmon, which contain quite large amounts. Most of the high fluorine items are not eaten daily, as are potatoes and bread. Nevertheless they would be expected to produce a marked increase in the consumption of fluorine on the particular days on which they are ingested. Hence the human daily consumption of food fluorine can vary widely and a large proportion of the total food fluorine may be consumed in sporadic doses. The results of an experiment in rats to investigate this point are now reported.

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The investigation of the rate of accumulation of fluorine in the body during the first six months revealed that the concentration of fluorine in the total body ash reached a constant value sooner than either the total body fluorine or the fluorine per unit of body weight. This suggested the possibility that the total body ash might exercise an effect on the rate at which fluorine would be stored on a given dietary intake. Results of an experiment to test this point by restricting calcium intake are reported below.

Experimental

The method of obtaining representative samples of entire rat carcasses and the analytical procedure used for the determination of fluorine have been described previously (1).

Experiment I—Rate of Fluorine Storage

This experiment was devised to determine the rate of storage of fluorine during a six-month feeding period. Three groups of 35 rats each were placed on rations containing 0.0, 0.5, and 1.0 mgm. of added fluorine per 100 gm. of basal ration. The basal ration contained about 0.02 mgm. of fluorine per 100 gm. The fluorine was added in the form of sodium fluoride ground up with an amount of casein such that 1 gm. of the mixture contained 5 mgm. of fluorine. Each group of 35 rats consisted of five litters of seven rats, and in each group the rats were matched for sex. The rats were 50 to 60 gm. in weight and about one month of age at the start of the experiment. The rations were fed ad libitum and a record was kept of the amount consumed. Initial controls of five rats (one of each litter) from each of the three groups were sacrificed and analyzed at the beginning of the feeding period. Subsequently, at monthly intervals for six months, additional sets of five rats from each group were sacrificed. They were analyzed for total fluorine and for total ash.

The results of this experiment are recorded in Table I. They have been corrected for the fluorine found in the initial controls. This was in the order of .05 mgm. in the total rat carcass. In the first part of the table is shown the total fluorine found, expressed as mgm., in the total rat carcass. Each result is the mean value of five rats. It can be seen that there was a continuing storage of fluorine over the entire period at both levels of fluorine intake tested. In the next portion of the table the results are expressed as mgm. of fluorine per 100 gm. of rat carcass. When calculated in this manner the diluting effect of growth is introduced. It was found that the actual concentration of fluorine in the rats' bodies was still increasing at the end of six months, although there was a tendency to level off, particularly at the 0.5 mgm. % level.

Finally the results are expressed as mgm. of fluorine per gm. of total body ash. When the results are calculated in this manner we see that the fluorine concentration in the ash reached a constant value at two to three months in

TABLE I

FLUORINE RETENTION BY RATS

Ration	1 month	2 months	3 months	4 months	5 months	6 months
As mgm. F in total rat carcass						
Basal	0.10	0.23	0.40	0.36	0.43	0.56
0.5 mgm. % F	1.01	1.94	2.74	2.55	3.51	3.99
1.0 mgm. % F	1.55	2.64	4.46	5.71	6.62	7.21
As mgm. F per 100 gm. of rat carcass, wet weight						
Basal	0.02	0.07	0.10	0.07	0.10	0.15
0.5 mgm. % E	0.59	0.73	0.89	0.92	1.31	1.40
1.0 mgm. % F	0.69	1.18	1.30	1.84	2.13	2.43
As mgm. F per gm. of ash						
Basal	0.01	0.02	0.03	0.02	0.02	0.03
0.5 mgm. % F	0.24	0.30	0.37	0.32	0.36	0.37
1.0 mgm. % F	0.34	0.44	0.50	0.59	0.70	0.70

Note: Each result is the mean of five animals and is corrected for the fluorine in the control group.

rats on the 0.5 mgm. % fluorine diet, and at five months in rats on the 1 mgm. % ration. The ultimate concentration of fluorine in the ash, when corrected for that found in the rats on the basal ration, was almost exactly proportional to the fluorine added to the ration. Thus an addition of 0.5 mgm. of fluorine per 100 gm. of ration increased the fluorine in the ash by 0.34 mgm. per gm. above the basal, while the addition of double the fluorine to the ration almost exactly doubled the fluorine retained in the ash, raising the value to 0.67–0.68 mgm. per gm., after correction was made for the basal value.

Experiment 2—Effect of Continuity of Fluorine Ingestion

This experiment was devised to test the effect of continuity of ingestion on the retention of dietary fluorine. Twenty-four rats, 50 to 60 gm. each, were divided into three groups matched for litter and sex. All rats were housed in individual cages. Group I was the control group and was given the standard animal room ration ad libitum. Group II was given the animal room ration to which 1 mgm. of fluorine, as sodium fluoride, had been added per 100 gm. of ration. The rats were allowed this ration ad libitum but each week the

amount consumed by each rat was determined and the fluorine consumption for the week was calculated. Group III was given the standard ration, without added fluorine, ad libitum. On one day each week the food was removed from the cage overnight. On the following morning each rat was given the amount of fluorine consumed by its litter-mate in Group II. This was mixed with a few grams of the regular ration. The rats, having fasted overnight, consumed the food quickly and completely. They were then given the regular ration again for the ensuing week. This regime was continued for a period of eight weeks, at the end of which time the rats were sacrificed and analyzed for fluorine.

TABLE II

Group	Added fluorine consumed, mgm. F/rat	Carcass fluorine found, mgm. F/rat	Added fluorine retained, mgm. F/rat	% of added fluorine retained
I. Control	0	0.12	0	—
II. Fluorine mixed with ration fed ad libitum	5.1	1.90	1.78	35
III. Fluorine fed once weekly	5.1	1.85	1.73	34

The results are shown in Table II. The retention of dietary fluorine was the same in both Groups II and III. Hence irregularity of ingestion of fluorine, to the extent of these experimental conditions, has no effect on the amount retained.

Experiment 3—Effect of the Body Ash on Fluorine Retention

This experiment was designed to test the effect of limiting the total body ash on the retention of dietary fluorine. Thirty-two rats of 50–60 gm. weight were divided into four groups of eight rats each, matched for litter and sex. Group IA was placed on the basal ration alone. This was the low calcium diet of E. Chant Robertson (2) and contained 14.5 mgm. % of calcium and 0.03 mgm. % of fluorine. Young rats fed this ration for four weeks have been shown to retain only about 50 mgm. of calcium compared to 1 gm. when fed a normal ration for the same period of time. Group IB received the same basal ration to which had been added 1 mgm. % of fluorine. Group IIA received the basal ration made up to contain 1% calcium by substitution of calcium carbonate, weight for weight, for a portion of the starch. Group IIB received the same ration as Group IIA, with the addition of 1 mgm. % fluorine. In both cases the fluorine was added as the stock sodium fluoride – casein mixture. All rations were fed ad libitum for eight weeks, while records were kept of the actual consumption. The rats were then sacrificed and analyzed.

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TABLE III

THE EFFECT OF DIETARY CALCIUM ON THE RETENTION OF FLUORINE

	I Low calcium	II High calcium
Added fluorine consumed, mgm. per rat	3.56	4.29
Added fluorine retained, mgm. per rat	1.38	1.32
% of added fluorine retained	41.5	30.8
Total ash, gm. per rat	1.92	4.24
Fluorine as mgm. per gm. of ash	0.77	0.35

The results are presented in Table III. In each case the result reported has been corrected for the corresponding basal control. It can be seen that the percentage retention of the added fluorine was considerably higher in the low calcium group than in the high calcium group. This was due to a decreased consumption of fluorine by the low calcium group, as the amount retained was about the same. The values are also given of the total ash and the fluorine expressed as mgm. per 100 gm. of ash. The total ash was less than half in the low calcium groups and the fluorine content was more than double. Despite this higher concentration of fluorine the percentage retention was better than that in rats on the high calcium diet, and the better even than the 35% retention observed in Experiment 2.

Discussion

In Experiment 1, the fluorine concentration of the total ash was found to reach a constant value at a time when the rats were still growing at a fairly rapid rate. This was particularly true when 0.5 mgm. % of fluorine was present in the ration. When a constant concentration of fluorine in the ash was attained, i.e. after two to three months on the 0.5 mgm. % ration or five months on the 1 mgm. % ration, further increases in the total carcass fluorine took place only at the rate at which the total body ash increased with growth. This could explain the greatly diminished rate of retention of dietary fluorine in adult rats, compared to that in young rats that has been observed by both ourselves (1) and Zipkin and McClure (3).

The observed relationship between the fluorine retention and ash leads one to the speculation that the total amount of fluorine that would be retained on a given dietary intake may be affected by the ash content of the body as well as by the amount of fluorine ingested. One would therefore expect to find that removal of calcium from the ration of young rats would decrease the retention of fluorine by preventing the normal increase in ash with growth. It was this consideration which led us to perform Experiment 3. However, we found a higher percentage retention of ingested fluorine when calcium was almost entirely absent from the ration, the fluorine attaining a concentration in the ash somewhat greater than twice that in the ash of rats on the high calcium ration. The interpretation of this finding is not entirely clear as

there is the complicating factor that the higher concentration of calcium in the intestinal tract of the rats on the high calcium ration probably reduced the amount of fluorine absorbed from the intestinal tract by precipitation of the ingested fluorine. Thus, while the retention of *ingested* dietary fluorine was less from the high calcium ration, the retention of *absorbed* fluorine may have been as high or higher than it was in rats on the low calcium ration. Further investigation of this point is contemplated.

Zipkin and McClure (3) observed that the total fluorine in rats' bones attained a plateau after the consumption of 10 p.p.m. (1 mgm.%) of fluorine in drinking water for 300 days. It has been our experience that, in order for rats to consume an equal amount of fluorine from drinking water or from food, the fluorine concentration in the food must be about twice that in the water. (See Table V (1).) Hence a level of 1 mgm. % in the water would lead to about the same total fluorine consumption as 2 mgm. % in the ration. We found that the fluorine, expressed as mgm. per gm. of ash, levelled off at two to three months with 0.5 mgm. % fluorine in the ration and at five months with 1.0 mgm. % in the ration. It might therefore be expected that it would require about nine months to reach a plateau with 2 mgm. % in the ration, a figure that corresponds well with the result of Zipkin and McClure (3).

The observation that the fluorine content of the bones became constant at the same age that the rats stopped growing rapidly, together with the additional observation that the retention of fluorine by adult rats was low, even though they had not previously been exposed to fluorine, led Zipkin and McClure (3) to conclude that the fluorine concentration in the bone ash reached a constant value owing to the cessation of growth. We have observed a stabilization of the fluorine concentration in ash at ages when the rats are still growing rapidly. It is probably because Zipkin and McClure chose a higher level of fluorine ingestion, requiring a longer time to reach a plateau, that they did not make a similar observation.

Conclusions

When fluorine is administered to rats over an extended period of time the retained fluorine will reach a constant concentration in the total ash. The ultimate concentration and the time required to reach it are proportional to the average rate of fluorine consumption. As the rat reaches maturity, so that the total ash becomes constant, the total body fluorine will also become constant. However, prevention of a normal increase in bone ash with age does not decrease the retention of the fluorine. The rate of consumption can fluctuate considerably, but the total retained fluorine in a given period will be the same provided the total dietary fluorine in the period was also the same.

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LECITHINASES IN NORMAL AND IN PATHOLOGICAL CEREBROSPINAL FLUIDS (CSF). I[1]

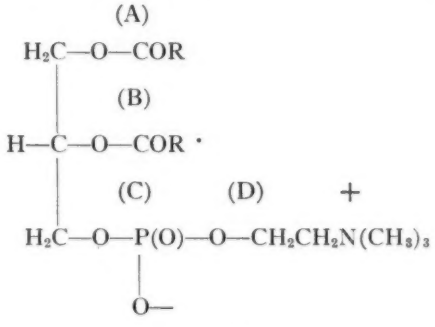
By E. Kovacs

Abstract

More than 480 CSF's were examined for lecithinase activity. No lecithin splitting was evident in definitely normal CSF. In 60% of the neurologically normal group moderate phospholipase level was demonstrated. Activity was found in a slightly higher number (64%) of the CSF's of meningitis patients. Of the poliomyelitis group 74% exhibited activity, with more uniformity in, and with higher, values than the first two groups. This finding is correlated with the pathological changes in the peripheral nervous system. The highest enzyme level was found in the syphilitic group, 80% of which showed positive assays. The CSF's of miscellaneous neurological and psychotic conditions occasionally exhibited intense lecithinase activity.

Introduction

Lecithinases (phospholipases, phophatidases) hydrolyze the phosphatides such as lecithins, cephalins, and others (26, 21, 10, 29, 36, 2). The action of at least four enzymes has been postulated since there are four ester linkages to be broken. These linkages have been arbitrarily assigned designations



Lecithin

A, B, C, and D; the enzymes concerned with their splitting have been named phospholipases and are similarly referred to as A, B, C, and D respectively (2, 7, 10, 30, 37). Phosphatide-splitting enzymes were demonstrated in body fluids (3), various tissue and organ extracts (21, 13), bacterial toxins (28), animal toxins (36, 35, 29), and in extracts of plants (30, 7, 14, 17). As far as we are aware, lecithinases have not been investigated systematically in the CSF. During the study of the diesterases (24, 25) we had occasion to detect the presence of lecithinase of CSF specimens. The results of these assays are reported in the present paper.

[1] *Manuscript received August 20, 1954.*

Contribution from the Department of Hygiene and Preventive Medicine, University of Toronto, Ont. This work was financed by a Mental Health Grant.

Methods

Purified phospholipid from ox-brain[2], consisting mainly of lecithin and containing only traces of other lipid material, was used routinely as substrate in 0.1% concentration. The stock solution in chloroform was kept in a refrigerator. The required volumes were emulsified in 0.2 *M* borate-buffer, pH 720 ± of Palits (22); after evaporation of the solvent, almost to dryness, at 50° C., portions of warm (50° C.) buffer were added under constant agitation. The emulsion was stable for weeks at 4° C. and gave a zero blank for inorganic phosphorus and a low value for total acid-soluble phosphorus.

Centrifuged, fresh, or freshly frozen CSF (0.2 ml.) was mixed with 10 ml. substrate; 0.2 ml. of 0.1% calcium chloride was added as activator, and the system incubated in glass-stoppered or plastic-capped bottles, routinely, for 24 hr. at 37° C. Controls were similarly incubated. In the substrate-control, CSF was replaced by physiological saline; in the CSF-control, buffer was substituted for substrate. Activator and one drop of toluene, as preservative, were added to the system and to the controls. A sterile technique was strictly observed and sterility tests were made from time to time on the incubated mixtures.

The method of Fiske and Subbarow as modified by King (21) served for inorganic phosphorus determination. Deproteinization was carried out, as previously described (23). No. 5 phosphorus-free Whatman filter paper was used for filtration. Duplicate determinations of aliquots of the filtrate were read on a Klett-Summerson photocolorimeter and the readings occasionally were checked on a Beckman D.U. spectrophotometer. Total acid-soluble phosphorus was determined on the same filtrate by King's method (21) of perchloric acid digestion. Controls were similarly treated. The difference between the values of the system and those of the controls, expressed as inorganic or as total acid-soluble phosphorus in mgm. per 100 ml. of the sample, indicated the change due to lecithinase activity.

For the demonstration of other split products of lecithin the titration of free fatty acids (2, 10) and the detection of choline (32) were used in some experiments. To control the reactivity of the substrate, lecithinase A of snake venoms (cobra and Russel's viper venom)[3] and lecithinase C of *C. welchii* toxin[4] were used in every new batch of substrate. Synthetic lecithin[5] and a commercial preparation of animal lecithin[6] purified by us by the petrol ether – acetone method (10) were also tested as substrate in a small series of experiments.

Results

About 480 individual CSF's from patients with various clinical diagnoses were examined for lecithinase activity. The patients from which the speci-

[2] *Kindly supplied by Prof. C. C. Lucas, Banting and Best Department of Medical Research, University of Toronto.*
[3] *Courtesy of the Haffkine Institute, Bombay.*
[4] *Kindly supplied by Dr. Helen Plummer, School of Hygiene, University of Toronto.*
[5] *Kindly supplied by Prof. E. F. T. Baer, Banting and Best Department of Medical Research.*
[6] *Nutritional Biochemicals, Cleveland, Ohio.*

TABLE I

INORGANIC PHOSPHORUS AND TOTAL ACID SOLUBLE PHOSPHORUS IN THE SAME SYSTEMS

(Illustrative examples)

Diagnosis	Increase of inorganic phosphorus (mgm.%) 24 hr. at 37° C.	Increase of total acid-soluble phosphorus (mgm.%) 24 hr. at 37° C.
Normal specimen	0	0
Meningococcal meningitis specimen	2.4	11.0
Bulbar poliomyelitis specimen	4.0	9.5
Late latent syphilis specimen	0	7.0
Brain tumor specimen	1.1	19.1

mens were obtained fell into five clinical groups: normal and neurologically normal and those having bacterial meningitides of various etiology, poliomyelitis and other virus diseases, syphilis and neurosyphilis, and miscellaneous neurological conditions and psychoneuroses. Diagnoses by the hospital staff were rigorously respected in the grouping.

Table I shows a few illustrative examples presenting the change in inorganic and total acid-soluble phosphorus values in the same systems. There is a definite, sometimes striking difference between the two types of change, which is in agreement with the findings of King (21). The discrepancy in some instances between the inorganic and the organically bound phosphorus values,

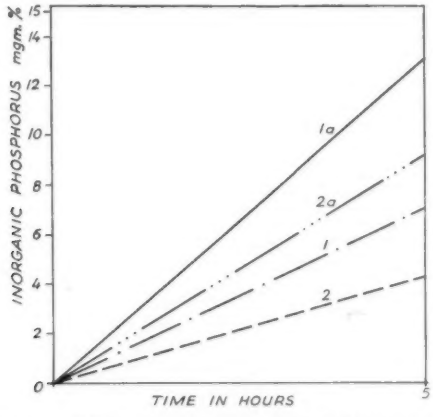


FIG. 1. Effect of Ca^{++} activation and of temperature.

1 ———— · ———— Neurosyphilis: 5 hr. at 47° C., no $CaCl_2$.
1a ———————— Neurosyphilis: 5 hr. at 47° C., 0.002% $CaCl_2$.
2 — — — — — — — — Manic-depressive psychosis: 5 hr. at 37° C., activated.
2a ———— · · ———— Manic-depressive psychosis: 5 hr. at 47° C., activated.

both of which indicate various steps in the hydrolysis of lecithin, suggests that under some circumstances the further hydrolysis of the fat-free compound is lacking.

Fig. 1 illustrates the effect of Ca^{++} activation. Similar enhancing of enzyme activity was obtained with Na^{+} and Mg^{++}, but in a lesser degree. The histochemical demonstration of lecithinases in tissues (16) at pH 9 is based on a principle similar to ours, namely the detection of the liberated lipoid phosphorus. As a matter of fact, a pH of 9 and other pH ranges were examined and found very suitable for our assays, but we chose purposely a pH range which is nearer to the milieu of the body.

TABLE II

LECITHINASES IN 450 CSF's

Group	Total number of specimens	Lecithinase activity present, %	Range of phosphorus changes, mgm.% 24 hr. 37° C.	Percent distribution in the various ranges
1. *a.* Normal	10	0	0	0
b. Neurologically normal	40	60	0– 6.7	0– 2 mgm.% 58% 2– 4 = 28 4– 6 = 11 6– 7 = 3
2. Meningitis	100	64	0– 6.0	0– 2 = 58 2– 4 = 34 4– 6 = 6 6– 8 = 2
3. Poliomyelitis	100	74	0– 8	0– 2 = 50 2– 4 = 26 4– 6 = 18 6– 8 = 6
4. Syphilis and neurosyphilis	100	80	0–27.8	0– 2 = 42 2– 4 = 19 4– 6 = 16 6– 8 = 8 8–10 = 5 10–12 = 3 12–14 = 1 14–16 = 2 16–18 = 1 18–20 = 1 20–22 = 1 26–28 = 1
5. Miscellaneous neurological and psychotic conditions	75* 25†	60	0–14.58	0– 2 = 58 2– 4 = 22 4– 6 = 11 6– 8 = 4 8–10 = 2 10–12 = 2 14–16 = 1

* *Neurological conditions.*
† *Psychotic conditions.*

The effect of temperature was striking. At 47° C. optimal results were obtained, but at 56° and 60° C. no inactivation of the enzymes occurred after five to six hours' incubation. This is in agreement with the findings of others concerning the thermostability of lecithinase A (26, 18). Synthetic lecithin substrate was more easily attacked by the CSF than were the natural products. Deep-freezing did not affect the enzyme activity of the CSF's, if they were frozen in fresh state.

Only the 24 hr. values were tabulated and presented. A longer incubation period resulted in comparatively higher values, but it was difficult to control the assays for more than two days in routine experiments owing to various factors, such as changes in the CSF, infection, etc.

Ten specimens of CSF taken from normal individuals did not exhibit any liberation of inorganic phosphorus (Table II, Group 1), when incubated even for periods up to 72 hr. The total acid-soluble phosphorus concentration was also unchanged, indicating the absence of phospholipase activity in these specimens (Table I, Group 1). On the other hand, 60% of the neurologically normal group exhibited some degree of lecithinase activity; the remainder behaved like the definitely normal group. The patients from which these CSF's derived were free of neurological symptoms and the samples, checked by routine laboratory tests in the hospitals, gave negative results, with one

TABLE III

LECITHINASES IN THE CSF

(Analysis of results in the two most active clinical groups)

Groups	Total number of specimens	Distribution of specimens with activity	Range of inorganic phosphorus changes, mgm.%
A. Poliomyelitis			
Non-paralytic form and suspects	47	33	0– 8
Paralytic form	30	25	0– 8
Bulbar form	20	15	0– 5.3
Encephalitic form	3	1	0– 1.32
B. Other virus diseases			
Lycytic choriomeningitis	10	2	0– 3
Infectious polyneuritis (Gillain-Barré syndrome)	2	1	0– 2
Mumps encephaiitis	7	3	0– 2.4
Measles encephalitis	5	1	0– 1
C. Syphilis, neurosyphilis			
Congenital	2	2	0– 2.3
Primary	5	3	0– 2.8
Secondary	5	4	0–19
Early, latent	20	14	0–15
Late, latent	20	15	0– 7
Neurosyphilis	40		0–28
Paretic type	5	47	0– 8
Tabetic type	5		0–21
Taboparetic type	5		0– 7

exception in which a moderate increase of cells was found. The CSF's from a patient with pharyngitis (6.7 mgm. %) and from one with lobar pneumonia (4.0 mgm. %) had two of the highest values in the 24 hr. incubation series.

High values occurred with greatest frequency in CSF from patients with pneumococcal meningitis. In a specimen from a convalescent meningitis patient no lecithinase activity was detected, while CSF samples from the same patient in the acute stage showed the presence of lecithin-hydrolyzing enzymes.

CSF specimens were examined from 100 patients with poliomyelitis of the non-paralytic, paralytic, bulbar, and encephalitic forms (Table II, Group 3, and Table III, Group A) and from 24 patients with miscellaneous virus diseases (lymphocytic choriomeningitis, Gillain-Barré syndrome, mumps and measles encephalitides) (Table III, Group B). Phospholipase activity was demonstrated in the vast majority of polio (74%) and in lesser degree in other virus disease samples (Table III). The CSF's of the paralytic form of poliomyelitis were especially rich in lecithinase. A few CSF specimens of fatal cases did not exhibit any activity. Free choline and fatty acids were occasionally demonstrated.

The syphilis and neurosyphilis group was the most homogeneous (Table II, group 4, and Table III, group C) in regard to occurrence and height of activity of lecithinase, as revealed by the results especially in the neurosyphilis subgroup. The results of the clinical subgroups presented in Table III demonstrate the highest activity in neurosyphilis. Many high values were found in the other clinical forms also. Titrations gave indication of the presence of free fatty acids at pH 3 (3), and free choline (32) was also demonstrated in a few neurosyphilis specimens that were investigated.

CSF's were examined from 75 patients with miscellaneous neurological conditions such as tumors, convulsive disorders, traumatic and vascular conditions, hydrocephalus, brain atrophy, polyneuritis, toxic encephalopathy, etc. In two demyelinating disease samples, moderately high lecithinase activity was observed. The highest value was found in porphyriasis (15 mgm. %) (Table I, group 5). Neoplasms and epilepsy samples exhibited, occasionally, some phosphatidase activity. Brain atrophy specimens (four CSF's) were active. In the psychoses group, a few CSF's of patients with an organic pathological basis had moderately high lecithinase levels.

Discussion

These assays are considered as a preliminary work. Because the volume of CSF used was often small, and the dilution at which it was tested was considerable, the degree of splitting obtained was often small, especially as the conditions were far from optimal. Thus the data give only a relative measure of the phosphatidase activity of these cerebrospinal fluids, not maximal values. The values, however, are real and infection could not be responsible for the higher results, because infected material was discarded, and the use of elevated temperature demonstrated clearly the enhanced phospholipase activity, without the possibility of bacterial multiplication.

We can only conclude that the observed lecithin splitting is the resultant of various, more or less specific, enzyme activities (2, 16, 37). Infection, metabolic disturbances, etc. may give rise to detectable enzyme levels, which may have their origin in the tissues (13, 3, 34). The role of lypolytic enzymes in degenerating and regenerating nerve tissue has been emphasized by some writers (19, 27). Higher activities observed in some meningitis specimens may be connected with the occasional participation of the brain tissue in the pathological process (33, 15).

Demyelination in the peripheral nerves in cases of acute poliomyelitis (8, 9) suggests some deeper connection with the frequent occurrence of phospholipase in the CSF. Such a connection is substantiated by the fact that the most intense activity is observed in paralytic polio samples. Exceptions in three fatal bulbar cases emphasize the need for time to produce these enzymes and also stress that some factors, like hypoxia (1), may influence the sensitive (10) ferment systems. The pathology of syphilis (4) and the part played by lecithins in the composition of the Wasserman antigen (31) suggest an abnormal lipid metabolism in the host. CSF's of neurosyphilitics were found to be highly neurolytic (23). The most frequent and intense lecithinase activity in similar specimens is therefore of special significance.

Products other than phosphorus split from lecithin were demonstrated, which means the complete breakdown of the complex molecule. We assume that at least one or two phospholipases have to act upon the substrate, thus opening the way for the action of diesterases (2, 11, 30, 35) and monoesterases (5, 6, 12), which bring the hydrolysis to completion. Some assays already have been made by us for the identification of the suspected lecithinases (A, B, and C), in well-defined small etiological groups of CSF's.

Acknowledgments

I wish to express my gratitude to the late Dr. D. T. Fraser, Head of the Department of Hygiene and Preventive Medicine, for his advice and criticism; and to the staffs of the Toronto hospitals for the CSF specimens kindly supplied. Mrs. E. Varkonyi has assisted in the technical work of most of these assays.

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